Gerinnungsproteasen als Therapie der Sepsis

Schuepbach, R A

Abstract: Als Äquivalent einer Habilitationsschrift werden drei Originalarbeiten zusammengefasst und eingereicht. Die aufgeführten Arbeiten beschreiben wie Gerinnungsproteasen via Protease aktivierbare Rezeptoren auf das Endothel wirken. Die Arbeiten zielen dahin, dass Gerinnungsfaktoren in Zukunft therapeutisch breiter und mit besserem Nutzen eingesetzt werden können. Quellenangabe der vorgestellten Publikationen: 1. Schuepbach RA, Feistritzer C, Brass LF, Riewald M. Activated protein C-cleaved protease activated receptor-1 is retained on the endothelial cell surface even in the presence of thrombin. Blood. 2008 Mar 1;111(5):2667-73. 2. Schuepbach RA*, Feistritzer C*, Fernandez JA, Griffin JH and Riewald M. Protection of Vascular Barrier Integrity by Activated Protein C Dependent on Protease-Activated Receptor-1. Thromb Haemost. 2009 Apr;101(4):724-33 3. Schuepbach RA and Riewald M. Clotting Factor Xa Cleaves PAR1 and Mediates Signaling Dependent on Binding to the Endothelial Protein C Receptor. J Thromb Haemost. 2010 Feb;8(2):379-88.

Gerinnungsproteasen als Therapie der Sepsis

Kumulative Habilitationsschrift

zur Erlangung der Venia Legendi an der Universität Zürich

von

Reto A. Schüpbach

Chirurgische Intensivmedizin

Universitätsspital Zürich

Rämistrasse 100

8091 Zürich

Zürich, Mai 2011

Als Äquivalent einer Habilitationsschrift werden drei Originalarbeiten zusammengefasst und eingereicht.

Die aufgeführten Arbeiten beschreiben wie Gerinnungsproteasen via Protease aktivierbare Rezeptoren auf das Endothel wirken. Die Arbeiten zielen dahin, dass Gerinnungsfaktoren in Zukunft therapeutisch breiter und mit besserem Nutzen eingesetzt werden können.

Quellenangabe der vorgestellten Publikationen:

1. **Schuepbach RA**, Feistritzer C, Brass LF, Riewald M. Activated protein C-cleaved protease activated receptor-1 is retained on the endothelial cell surface even in the presence of thrombin. Blood. 2008 Mar 1;111(5):2667-73.

2. **Schuepbach RA***, Feistritzer C*, Fernandez JA, Griffin JH and Riewald M. Protection of Vascular Barrier Integrity by Activated Protein C Dependent on Protease-Activated Receptor-1. Thromb Haemost. 2009 Apr;101(4):724-33

3. **Schuepbach RA** and Riewald M. Clotting Factor Xa Cleaves PAR1 and Mediates Signaling Dependent on Binding to the Endothelial Protein C Receptor. J Thromb Haemost. 2010 Feb;8(2):379-88.

Content

Attachements

Schuepbach RA, Feistritzer C, Brass LF, Riewald M. Activated protein C-cleaved protease activated receptor-1 is retained on the endothelial cell surface even in the presence of thrombin. Blood. 2008 Mar 1;111(5):2667-73.

Schuepbach RA*, Feistritzer C*, Fernandez JA, Griffin JH and Riewald M. Protection of Vascular Barrier Integrity by Activated Protein C Dependent on Protease-Activated Receptor-1. Thromb Haemost. 2009 Apr;101(4):724-33

Schuepbach RA and Riewald M. Clotting Factor Xa Cleaves PAR1 and Mediates Signaling Dependent on Binding to the Endothelial Protein C Receptor. J Thromb Haemost. 2010 Feb;8(2):379-88.

1. Introduction

Sepsis causes an estimated 240,000 deaths per year in the United States [1] and around 3000 deaths/year in Switzerland [2]. Despite enormous efforts to develop new drugs for treating sepsis, only one single compound has come to market so far, the recombinant human clotting factor activated protein C (APC) [3]. APC was found to be beneficial for patients with severe sepsis, however it has dose limiting side effects such as bleeding complications [4]. Therefore, it is crucial to understand the mode of action of APC in septic patients to come up with novel and better treatment strategies [5-7].

Almost a decade ago I became involved in studying how APC mediates protective effects. At that time it was generally assumed that anticoagulatory effects of APC might mediate benefits through prevention from microthrombosis [8, 9]. Nevertheless alternative non anticoagulant mechanisms of APC were proposed [10]: In vitro, APC was shown to cleave and activate the thrombin receptor, the protease activated receptor 1 (PAR1) in an EPCR dependent manner [11]. However the physiological relevance of this novel signaling pathway was controversially discussed. Arguments included the fact that the generation of APC requires the presence of thrombin in the first place [9]. It was argued that thrombin would have already activated all available PAR1 at the time when APC would become available [9].

In my studies my colleagues and I assumed that protease binding to co-receptors might affect the relative efficiency of thrombin and APC in cleaving PAR1 and we could show that in fact, APC was able to cleave and activate PAR1 and subsequently mediate endothelial barrier sealing effects despite the presence of thrombin [12]. Beyond cleavage of PAR1, APC-PAR1 could also be shown to mediate physiologically relevant effects in endothelial cells in the presence of thrombin. Thus we were able to give evidence, that the APC-PAR1 pathway was indeed a physiologically relevant clotting protease signaling pathway. Subsequent studies confirmed our findings [13]. Mice

studies further identified PAR1 as the target receptor for the high dose APC treatment which mediates many beneficial effects in the mouse injury model [6].

Next our group addressed the question if the high dose APC treatment in the mouse injury model reflects the situation of prolonged infusions of low dose APC which is used in humans suffering from severe sepsis. In humans APC treatment is achieved with APC plasma concentrations up to 100 times lower that the ones used in mice. Further the highly related receptors PAR3 and 4 show substantial functional differences between mouse and man [14]. We asked whether APC cleaves and activates PAR1 similarly in mouse and man and whether prolonged infusions of low dose APC still mediate PAR1 dependent beneficial effects in a mouse injury model [15]. Our studies could not relieve species differences, further corroborating that also in humans the APC-PAR1 signaling pathway mediates benefits in systemic inflammation such as sepsis.

While screening for ways to eventually dissect anticoagulant form signaling proprieties of APC we studied secondary side interactions between APC and substrates [16]. Further we looked at the binding interaction of APC's Gla domain to the endothelial protein C (co-) receptor (EPCR). Unexpectedly, we found that in addition to APC the structurally related clotting factor X and its activated form (FXa) also firmly bind EPCR [17]. Although it remains unclear whether the interaction of FXa and EPCR is of direct physiological importance, it might well explain some clinical observations. Only in patients with a severely altered clotting system (i.e. depleted clotting factors) therapeutically infused APC is efficient [18]. Given the very low concentrations of therapeutically infused APC (0.5nM) competition by FX (200nM) for EPCR binding might well explain the inefficiency of APC in patients with unaltered clotting system.

To summarize, these studies help to better understand the target mechanism of therapeutically infused APC in systemic inflammation in humans and have helped to design future mutants of APC which are currently under investigation in my laboratory and which will hopefully help to improve therapeutic effects of APC.

2. Results

2.1. Activated protein C-cleaved protease activated receptor-1 is retained on the endothelial cell surface even in the presence of thrombin.

Blood. 2008 Mar 1;111(5):2667-73 [12]

A corner stone of inflammatory diseases such as sepsis is the increased vascular permeability resulting in hypovolemic shock and upon intravenous volume therapy in interstitial edema [19]. Here we used our established *in vitro* model quantifying the endothelial barrier function [20]. EA.hy.926 endothelial cells were grown on a microporose membrane separating two compartments. Into the apical compartment which represents the intravascular room, Evans Blue labelled albumin was placed. To quantify the endothelial barrier function, the amount of dye passing through the endothelial layer was quantified in the bottom (interstitial) compartment. With this system we could show that APC can induce powerful barrier protective responses. Whereas endothelial barrier enhancing effects of APC have previously been shown to depend on binding of APC to the endothelial protein C (co-) receptor and cleavage of PAR1, it was controversially discussed if APC generated by thrombin still can act through PAR1 [8, 9]. In experiments where APC and thrombin were added together onto endothelial cells, we could show that APC still mediates barrier enhancing effects. And even when thrombin was added together with the non proteolytically active xymogen protein C the over time generated APC was still efficient in inducing barrier sealing effects, rising questions about the underlying mechanism.

Using cell surface immunoassays with conformation sensitive monoclonal anti-PAR1 antibodies we quantified cleavage and trafficking of endogenous expressed PAR1 on the endothelial cell surface following incubation with APC in the absence and presence of thrombin. Incubation with APC caused efficient PAR1 cleavage and upon co-incubation with thrombin APC still supported additional PAR1 cleavage. Thrombin-cleaved PAR1 rapidly disappeared from the cell surface whereas, unexpectedly, the APC-cleaved PAR1 remained and could be detected on the cell surface, even when thrombin at concentrations of up to 1 nM was also present.

These findings demonstrated for the first time directly that APC can mediate biological effects under physiological conditions where thrombin is also present. Further, they provide a molecular model on how APC acts on PAR1 by suggesting a distinct PAR1 population on endothelial cells with specific trafficking proprieties witch seems susceptible to APC but not to thrombin.

2.2. Protection of Vascular Barrier Integrity by Activated Protein C Dependent on Protease-Activated Receptor-1

Thromb Haemost. 2009 Apr;101(4):724-33 [15]

The mode of action of therapeutically infused APC in patients with sepsis is controversially discussed [21]. Mouse models are of key importance to clarify target mechanisms of drugs in translational research but species differences may limit conclusions. We analyzed whether mouse APC can cleave, activate and induce signaling through murine PAR1. In newly established mouse models we tested if long term infusion with low does APC, as it is used in humans, prevents from vascular leakage in a mouse model and if beneficial effects can be explained by APC signaling through the PAR1 pathway in mice. Cell surface immunoassays demonstrated efficient cleavage of endogenous murine endothelial PAR1 by either murine or human APC. Of importance, there were no species differences detected in how APC cleaves PAR1 and in regard to the specific receptor kinetics of APC-cleaved PAR1. Pharmacological concentrations of APC of either species had powerful barrier protective effects on cultured murine endothelial cells and required PAR1 cleavage. *In vivo*, vascular endothelial growth factor-mediated hyperpermeability in the skin of wild type mice was reduced by either endogenously generated or by directly infused recombinant mouse APC. Similarly long term infusions of low dose APC, as used in humans with sepsis [3], reduced lung edema formation in mice suffering from systemic inflammation induced by endotoxin in a PAR1 dependent manner.

Our results directly show that murine APC cleaves and signals through PAR1 in murine endothelial cells. Further we could not find any evidence for species differences between mouse and man in terms of how APC cleaves and activates PAR1. Prolonged infusions of low dose APC reduce the vascular permeability in mouse models and PAR1 plays a major role in mediating these effects.

Together these *in vitro* and *in vivo* data support the paradigm that PAR1 contributes to protective effects of APC on vascular barrier integrity in sepsis.

2.3. Clotting Factor Xa Cleaves PAR1 and Mediates Signaling Dependent on Binding to the Endothelial Protein C Receptor

J Thromb Haemost. 2010 Feb;8(2):379-88 [17]

Coagulation is intrinsically tied to inflammation, and both pro-inflammatory and anti-inflammatory responses are modulated by coagulation protease signaling through protease-activated receptor-1 (PAR1). Activated factor X (FXa) can elicit cellular signaling through PAR1 [22], but little is known about the role of cofactors in this pathway.

Endothelial protein C receptor (EPCR) supports PAR1 signaling by APC. So far it remains unclear if other Gla-domain containing proteases such as FXa compete with APC for EPCR binding and subsequent activation of PAR1. We were especially interested if FX can compete with APC for EPCR, given the high physiological plasma concentration of FXa of around 200nM.

We tested if FXa binds to EPCR in endothelial cells which i) either natively expressed EPCR or ii) in endothelial cells in which EPCR expression was down regulated or in CHO-K1 cells which iii) either did not or iv) stably overexpress EPCR. We found that FXa binds to both natively and overexpressed EPCR with proprieties comparable to protein C or APC. Further we found that FXa competes for EPCR binding with protein

C, APC and clotting factor VII but not with protein S which also shares a high homology to all EPCR binding clotting proteases and also contains a Gla-domain.

PAR1 cleavage by FXa as analyzed with conformation-sensitive antibodies and a tagged PAR1 reporter construct was strongly enhanced if EPCR was available. Anti-EPCR failed to affect the tissue factor-dependent activation of FX, but high concentrations of FXa decreased EPCR-dependent protein C activation. Most importantly, the FXa-mediated induction of Erk1/2 activation, expression of the transcript for connective tissue growth factor and barrier protection in endothelial cells required binding to EPCR.

Taken together, these results demonstrate that EPCR plays an unexpected role in supporting cell surface recruitment, PAR1 activation, and signaling by FXa. Further the similar binding proprieties of FXa and protein C or APC together with evidence for direct competition for EPCR binding provide a model for why therapeutic infusions of low dose APC were efficient only in patients with severely depleted clotting factor concentrations. In patients having unaffected clotting factor concentrations, the physiological concentration of FX (200nM) might well compete with the therapeutic concentration of APC (0.5nM) for their target co-receptor EPCR.

3. Literature

1. Angus, D.C., et al., *Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care.* Crit Care Med, 2001. **29**(7): p. 1303-10.
2. Schmid, A., et al., *Burden of illness imposed by severe sepsis in Switzerland.* Swiss Med Wkly, 2004. **134**(7-8): p. 97-102.
3. Bernard, G.R., et al., *Efficacy and safety of recombinant human activated protein C for severe sepsis.* N Engl J Med, 2001. **344**(10): p. 699-709.
4. Khan, A., et al., *Prevalence of serious bleeding events and intracranial hemorrhage in patients receiving activated protein C: a systematic review and meta-analysis.* Respir Care, 2010. **55**(7): p. 901-10.
5. Guo, H., et al., *Neuroprotective activities of activated protein C mutant with reduced anticoagulant activity.* Eur J Neurosci, 2009. **29**(6): p. 1119-30.
6. Kerschen, E.J., et al., *Endotoxemia and sepsis mortality reduction by non-anticoagulant activated protein C.* J Exp Med, 2007. **204**(10): p. 2439-48.
7. Di Cera, E., *Know your APC.* Blood, 2009. **113**(23): p. 5699-700.
8. Ludeman, M.J., et al., *PAR1 cleavage and signaling in response to activated protein C and thrombin.* J Biol Chem, 2005. **280**(13): p. 13122-8.
9. Esmon, C.T., *Is APC activation of endothelial cell PAR1 important in severe sepsis?: No.* J Thromb Haemost, 2005. **3**(9): p. 1910-1.
10. Ruf, W., *Is APC activation of endothelial cell PAR1 important in severe sepsis?: Yes.* J Thromb Haemost, 2005. **3**(9): p. 1912-4.
11. Riewald, M., et al., *Activation of endothelial cell protease activated receptor 1 by the protein C pathway.* Science, 2002. **296**(5574): p. 1880-2.
12. Schuepbach, R.A., et al., *Activated protein C-cleaved protease activated receptor-1 is retained on the endothelial cell surface even in the presence of thrombin.* Blood, 2008. **111**(5): p. 2667-73.
13. Russo, A., et al., *Caveolae are required for protease-selective signaling by protease-activated receptor-1.* Proc Natl Acad Sci U S A, 2009. **106**(15): p. 6393-7.
14. Nakanishi-Matsui, M., et al., *PAR3 is a cofactor for PAR4 activation by thrombin.* Nature, 2000. **404**(6778): p. 609-13.
15. Schuepbach, R.A., et al., *Protection of vascular barrier integrity by activated protein C in murine models depends on protease-activated receptor-1.* Thromb Haemost, 2009. **101**(4): p. 724-33.
16. Mosnier, L.O., et al., *Hyper-antithrombotic, non-cytoprotective Glu149Ala-activated protein C mutant.* Blood, 2009.
17. Schuepbach, R.A. and M. Riewald, *Coagulation factor Xa cleaves protease-activated receptor-1 and mediates signaling dependent on binding to the endothelial protein C receptor.* J Thromb Haemost, 2010. **8**(2): p. 379-88.
18. Abraham, E., et al., *Drotrecogin alfa (activated) for adults with severe sepsis and a low risk of death.* N Engl J Med, 2005. **353**(13): p. 1332-41.
19. Lee, W.L. and A.S. Slutsky, *Sepsis and endothelial permeability.* N Engl J Med, 2010. **363**(7): p. 689-91.
20. Feistritzer, C., et al., *Protective signaling by activated protein C is mechanistically linked*

to protein C activation on endothelial cells. J Biol Chem, 2006. **281**(29): p. 20077-20084.
21. Toussaint, S. and H. Gerlach, *Activated protein C for sepsis.* N Engl J Med, 2009. **361**(27): p. 2646-52.
22. Feistritzer, C., R. Lenta, and M. Riewald, *Protease-activated receptors-1 and -2 can mediate endothelial barrier protection: role in factor Xa signaling.* J Thromb Haemost, 2005. **3**(12): p. 2798-805.

CURRICULUM VITAE
RETO ANDREAS SCHUEPBACH

Personal Data:

Date of Birth:	February 5th 1971
Place of birth:	Landiswil (BE)
Marital Status:	Married to PD Dr. med. Annelies Zinkernagel, PhD, MSc
Children:	2 daughters, born 08.07.2004 and 23.07.2007

Business Address:	University Hospital Zürich Surgical Intensive Care, HOF B 110 Rämistrasse 100 CH-8091 Zurich Switzerland Tel. 044-255 9261 Tel. 044-255 1111 (Switch board University Hospital Zürich) E-mail: reto.schuepbach@usz.ch
Home Address:	Attenhoferstrasse 12 8032 Zurich, Switzerland Tel. 044-268 5880 E-mail: reto.schuepbach@gmx.net

Education and Professional Degrees:

05/2008	Master of Advanced Studies in Clinical Research at the University of California, San Diego (USA)
6/2007	Graduation from Clinical Research Enhancement through Supplemental Training (CREST) at the University of California, San Diego (USA)
1/2005	Board Certificate in Intensive Care, Switzerland (FMH)
5/2002	Board Certificate in Internal Medicine, Switzerland (FMH)
1997	M.D. Thesis: Bioassay for hamster macrophage chemotaxis: application to study particle-lung interactions (University of Bern, Switzerland)
1996	Swiss Federal Diploma of Physician (MD degree)
1990 - 1996	Medical School, University of Bern, Switzerland
1990	Matura summa cum laude (2. Award of the city of Bern) Bern Switzerland

Professional Record:

Clinical work:

Since 9/2009	Attending physician (Oberarzt), Chirurgische Intensivmedizin, University Hospital Zurich, Switzerland
1/2003 - 3/2005	ICU Fellow, Internal Medicine, Cardio-Surgery and Trauma Care, University Hospital Zurich, Switzerland
6/2002 - 12/2002	Resident, Anesthesiology, University Hospital Zurich, Switzerland
2/2002 - 5/2002	Resident, Nephrology, University Hospital Zurich, Switzerland
10/2000 - 2/2002	Resident, Internal Medicine, University Hospital Zurich, Switzerland
10/1998 - 9/2000	Resident, Internal Medicine, Zieglerspital, Bern, Switzerland
4/1998 - 9/1998	Resident, ICU for Internal Medicine and Surgery, Regionalspital Biel, Switzerland
4/1997 - 4/1998	Resident, Surgical Department and Emergency Room, Regionalspital Biel, Switzerland

Laboratory work:

Since 9/2009	Independent Laboratory Group Leader, Zentrum für Klinische Forschung, Chirurgische Intensivmedizin, University Hospital Zurich, Switzerland
7/2005 - 6/2009	Research Associate at the Scripps Research Institute, Department of Immunology, La Jolla, California, USA
10/1996 - 3/1997	Full time laboratory work as graduate student in the laboratory of cytology, Anatomy, University of Bern, Switzerland
1992 - 9/1996	Part time (cumulative 11 month full time) laboratory work as a student in the laboratory of cytology, Anatomy, University of Bern, Switzerland

University lectures:

Since 9/2009	Lectures, seminars and clinical teaching of medical students
Since 1/2010	Organization and moderation of weekly teaching sessions in Intensive Care Medicine: "Montagsweiterbildung Intensivmedizin" with broadcasting to over 30 hospitals in and around Zurich
2005-2009	Vascular biology, The Scripps Research Institute, La Jolla, CA, USA

Awards and Personal Grants

11/2011-11/2014	Swiss National Foundation, SCORE/Ambizione subsidy
6/2010	Huggenberger-Bischoff Stiftung, Preis für Krebsforschung
2010	Projekt- und Personenförderung durch die Universität Zurich, Switzerland
7/2007 - 6/2008	Swiss Foundation for Medical-Biological Grants (SSMBS),
7/2008 - 6/2009	Mach-Gaensslen Stiftung, post doctoral fellowship post doctoral fellowship
7/2006 - 6/2007	University of Zurich, Switzerland; Stiefel-Zangger-Stiftung, post doctoral fellowship
7/2005 - 6/2006	Swiss National Foundation, post doctoral fellowship grant# PBBBE-108544
7/2005	Ida & Herzog Egli Stiftung, grant # 255

Project Grants:

8/2010	Olga Maiyenfisch Stiftung, Projektgrant
6/2010	Ida-de Pottèrre-Leupold Fonds, Projektgrant
4/2010-4/2012	SNF Projektgrant, Importance of the M1 protein structure for virulence of group A streptococcus #310030-130748 (Co-Investigator)
9/2009 - 8/2010	Projektförderung, Hartmann Müller-Stiftung, grant#1314

List of Publications

Reto Andreas Schuepbach, MD, MSc

Peer-reviewed articles:

1. Meierhans R, Brandi G, Fasshauer M, Sommerfeld J, **Schüpbach R**, Béchir M, Stover JF. Arterial lactate above 2 mM is associated with increased brain lactate and decreased brain glucose in patients with severe traumatic brain injury. Ahead of publication, Minerva Anestesiol. 2011 Nov 5. PubMed PMID: 21971438.

2. Zinkernagel AS, Hruz P, Uchiyama S, von Köckritz-Blickwede M, **Schuepbach RA**, Hayashi T, Carson DA, Nizet V. Importance of Toll like receptor 9 in host defense against M1T1 Group A Streptococcus infections. J Innate Immun. 2012;4(2):213-8. Epub 2011 Aug 19.

3. Quiblier Ch, Zinkernagel AS, **Schuepbach RA**, Berger-Baechi B, Senn MM. Contribution of SecDF to Staphylococcus aureus resistance and expression of virulence factors. BMC Microbiol. 2011 Apr 12;11(1):72.

4. **Schuepbach RA**, Bestmann L, Béchir M, Fehr J, Bachli EB. High prevalence of iron deficiency among educated hospital employees in Switzerland. Int J Biomed Sci. 2011 Jun 15; 7(2):131-136.

5. **Schuepbach RA**, Velez K, and Riewald M. Activated protein C upregulates procoagulant tissue factor activity on endothelial cells by shedding TFPI's Kunitz 1 domain. Blood. 2011 Jun 9;117(23):6338-46.

6. Bachli EB, Bösiger J, Béchir M, Stover JF, Stocker R, Maggiorini M, Renner EL, Müllhaupt B, **Schuepbach RA**. Thromboelastography to Monitor Clotting / Bleeding Complications in Patients Treated with the Molecular Adsorbent Recirculating System (MARS). Crit Care Res Pract. 2011;2011:313854.

7. Oberkofler CE, Stocker R, Raptis DA, Stover JF, **Schuepbach RA**, Müllhaupt B, Dutkowski P, Clavien PA and Bechir M. Same quality – higher price? The paradox of allocation: the first national single center analysis after the implementation of the new Swiss transplantation law: the ICU view. Clin Transplant. 2010 Nov 26. Ahead of publication.

8. Oberkofler CE, Duttkowski P, Stocker R, Stover JF, **Schuepbach RA**, Clavien PA, Bechir M. Model of end stage liver disease (MELD) score greater than 23 predicts length of stay in the ICU but not mortality in liver transplant recipients. Crit Care. 2010;14(3):R117.

9. Bosshart M, Stover JF, Stocker R, Asmis LM, Feige J, Neff TA, **Schuepbach RA**, Cottini SR, Bechir M. Two different hematocrit detection methods: Different methods, different results? BMC Res Notes. 2010, Mar 9, 3:65.

10. **Schuepbach RA** and Riewald M. Clotting Factor Xa Cleaves PAR1 and Mediates Signaling Dependent on Binding to the Endothelial Protein C Receptor. J Thromb Haemost. 2010 Feb;8(2):379-88.

11. **Schuepbach RA***, Feistritzer C*, Fernandez JA, Griffin JH and Riewald M. Protection of Vascular Barrier Integrity by Activated Protein C Dependent on Protease-Activated Receptor-1. Thromb Haemost. 2009 Apr;101(4):724-33.
* these authors contributed equally

12. Mosnier LO, Zampolli A, Kerschen EJ, **Schuepbach RA**, Banerjee Y, Fernandez JA, Yang XV, Riewald M, Weiler H, Ruggeri ZM and Griffin JH. Hyperantithrombotic, noncytoprotective Glu149Ala-activated protein C mutant. Blood. 2009 Jun 4;113(23):5970-8. Epub 2009 Feb 24.

13. **Schuepbach RA**, Feistritzer C, Brass LF, Riewald M. Activated protein C-cleaved protease activated receptor-1 is retained on the endothelial cell surface even in the presence of thrombin. Blood. 2008 Mar 1;111(5):2667-73.

14. Peyssonnaux C, Zinkernagel AS, **Schuepbach RA**, Rankin E, Haase V, Vaulont S, Nizet V and Johnson RS. The Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIF). J Clin Invest. 2007 Jul;117(7):1926-32.

15. Bachli EB*, **Schuepbach RA***, Maggiorini M, Stocker R, Müllhaupt B, Renner EL. Artificial Liver Support with the Molecular Adsorbent Recirculating System (MARS): Activation of Coagulation and Bleeding Complications. Liver Int. 2007 May;27(4):475-84.
* these authors contributed equally

16. Feistritzer C, **Schuepbach RA**, Mosnier LO, Bush LA, Di Cera E, Griffin JH, Riewald M. Protective signaling by activated protein C is mechanistically linked to protein C activation on endothelial cells. J Biol Chem. 2006 Jul 21;281(29):20077-84.

17. Geiser M, **Schupbach R**, Waber U, Gehr P. Bioassay for hamster macrophage chemotaxis: application to study particle-lung interactions. Cell Mol Life Sci. 1998 Feb;54(2):179-85.

Case reports:

18. **Schuepbach RA**, Meili EO, Schneider E, Peter U, Bachli EB. Lepirudin therapy for thrombotic complications in congenital afibrinogenaemia. Thromb Haemost. 2004 May;91(5):1044-6.

19. Kronenberg A.*, **Schupbach R**.*, Schuknecht B., Bossart W., Weber R., Gilden DH., Speck RF. Multifocal Vasculopathy Due to Varicella-Zoster Virus (VZV): Serial Analysis of VZV DNA and Intrathecal Synthesis of VZV Antibody in Cerebrospinal Fluid. Clin Infect Dis. 2002 Aug 1;35(3):330-3. Epub 2002 Jul 8.
* these authors contributed equally

Reviews:

20. Riewald M, **Schuepbach RA**. Protective signaling pathways of activated protein C in endothelial cells. Arterioscler Thromb Vasc Biol. 2008 Jan;28(1):1-3.
    Impact factor 7.24

Activated protein C–cleaved protease activated receptor-1 is retained on the endothelial cell surface even in the presence of thrombin

Reto A. Schuepbach,[1] Clemens Feistritzer,[1] Lawrence F. Brass,[2] and Matthias Riewald[1]

[1]Department of Immunology, Scripps Research Institute, La Jolla, CA; and [2]University of Pennsylvania, Philadelphia

Activated protein C (APC) signals in endothelial cells ex vivo through protease activated receptor-1 (PAR1). However, it is controversial whether PAR1 can mediate APC's protective effects in sepsis because the inflammatory response results in thrombin generation and thrombin proteolytically activates PAR1 much more efficiently than APC. Here we show that APC can induce powerful barrier protective responses in an endothelial cell monolayer in the presence of thrombin. Using cell surface immunoassays with conformation sensitive monoclonal anti-PAR1 antibodies we analyzed cleavage of endogenous PAR1 on the endothelial cell surface by APC in the absence and presence of thrombin. Incubation with APC caused efficient PAR1 cleavage and upon coincubation with thrombin APC supported additional PAR1 cleavage. Thrombin-cleaved PAR1 rapidly disappeared from the cell surface whereas, unexpectedly, the APC-cleaved PAR1 remained and could be detected on the cell surface, even when thrombin at concentrations of up to 1 nM was also present. Our findings demonstrate for the first time directly that APC can generate a distinct PAR1 population on endothelial cells in the presence of thrombin. The data suggest that different trafficking of activated PAR1 might explain how PAR1 signaling by APC can be relevant when thrombin is present. (Blood. 2008;111: 2667-2673)

Introduction

Recombinant human activated protein C (APC) has powerful protective effects in systemic inflammation that led to its approval to treat patients with severe sepsis.[1] Protein C (PC) is physiologically activated on the endothelial cell surface by the key procoagulant enzyme thrombin and APC down-regulates thrombin formation in a negative feedback loop.[2] However, this anticoagulant effect of APC is unlikely to explain its benefit in systemic inflammation because other anticoagulants with comparable effect do not improve survival in septic patients[3,4] or in animal models.[5]

More recently, APC signaling through protease activated receptor-1 (PAR1) emerged as an alternative mechanism for APC's beneficial effects.[6,7] In cultured endothelial cells PAR1 mediates protective effects of APC on gene expression,[6,8] survival,[9,10] and barrier integrity.[11] PAR1 has also been implicated in mouse models analyzing neuroprotective effects of APC in vivo.[9,12,13] PAR1 is a 7-transmembrane G-protein–coupled receptor that is enzymatically cleaved after Arg41 to expose a new extracellular N-terminus that acts as a tethered activating ligand.[14] PAR1 is the prototypical thrombin receptor and thrombin cleaves and activates PAR1 with high efficiency because it directly binds to PAR1 in an orientation that favors cleavage.[15] In contrast, APC needs to be recruited to a coreceptor, the endothelial protein C receptor (EPCR), to induce PAR1-dependent signaling[6] but APC remains less efficient than thrombin.[16] Systemic inflammation leads to generation of thrombin and in view of the relatively low efficiency of PAR1 activation by APC it has been argued that a role of APC-PAR1 signaling in sepsis is unlikely.[16,17]

Here we demonstrate that APC can mediate significant PAR1 cleavage even in the presence of thrombin. In contrast to thrombin-cleaved PAR1, the APC-cleaved PAR1 accumulates on the endothelial cell surface. The findings support the concept that PAR1 can mediate protective APC signaling in conditions where thrombin is also present.

Methods

Reagents and assays

Human thrombin was as described.[6,18] Human plasma–derived APC and PC were from Haematologic Technologies (Essex Junction, VT). Recombinant human wild-type APC and mutant APC S360A were a gift from Dr John Griffin (Scripps Research Institute, La Jolla, CA).[19] All experiments involving stimulation with APC included hirudin (Calbiochem, La Jolla, CA) unless indicated otherwise or if cells were coincubated with APC and thrombin. Control experiments demonstrated that hirudin alone had no effect in any of our assays. Brefeldin A was from Calbiochem. Monoclonal anti-PAR1 ATAP2, WEDE15, and SPAN11 were as described.[20,21] Monoclonal rat anti-EPCR RCR-92 (nonblocking) and RCR-252 (blocking) were provided by Dr Kenji Fukudome (Saga Medical School, Saga, Japan) and were used at 25 μg/mL.[22] Amidolytic assays for APC activity were as described previously.[23]

Cell culture, permeability assay, and surface immunoassays

EA.hy926 cells[24] and primary human umbilical vein endothelial cells (HUVEC; Cascade Biologics, Portland, OR) were cultivated and macromolecular monolayer permeability was analyzed in a dual chamber system using Evans blue-labeled bovine serum albumin (BSA) as described previously.[11] For cell-surface enzyme linked immunoassays (ELISA), the cells were fixed with 2% paraformaldehyde, blocked with 1% BSA and

Submitted September 17, 2007; accepted December 13, 2007. Prepublished online as *Blood* First Edition paper, December 17, 2007; DOI 10.1182/blood-2007-09-113076.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

probed with anti-PAR1 antibodies at 0.5 μg/mL for 30 minutes. A horseradish peroxidase (HRP)–coupled goat anti-mouse antibody and tetrametylbenzidine were used for spectrophotometric quantification of anti-PAR1 binding. To facilitate the comparison of different experiments the quantification of antibody binding was normalized. Specific blocking peptides TFLLRNPNDK (for ATAP2), KYEPFWEDEEKNES (for WEDE15), and NATLDPRSFFLR (for SPAN11) were custom made (Invitrogen, Carlsbad, CA) and all peptides completely blocked the respective specific anti-PAR1 staining. Residual unspecific staining was found to be unaffected by agonists and was subtracted to correct for background (0% staining). In all experiments nonagonist-treated cells were included and PAR1 staining in these cells was defined as 100%. The shown data were generated using EA.hy926 cells and results were confirmed in HUVECs. For immunofluorescence microscopy EA.hy926 cells were grown, fixed, and stained on glass cover slides using identical conditions as for ELISA. Alexa Fluor 488-coupled goat antimouse (Invitrogen) was used as the secondary antibody. The cover slides were extensively washed, mounted (Gelmount; Sigma-Aldrich, St Louis, MO), and immediately analyzed using an Olympus BX60 fluorescence microscope (Olympus, San Diego, CA).

Biotinylation of cell-surface proteins and Western blotting

After agonist incubation, proteases were quenched, EA.hy926 cells were washed twice on ice and kept at 4°C for all subsequent steps. Cell-surface proteins were biotinylated (0.2 mg/mL Sulfo-NHS-SS-biotin for 30 minutes; Pierce, Rockford, IL) before extraction in RIPA buffer supplemented with Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN), 20 μM bestatin, and 0.1 mg/mL phenylmethylsulfonyl fluoride. Biotinylated proteins were collected using streptavidin agarose (Invitrogen). Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, transferred to Immobilon-P-membranes (Millipore, Bedford, MA), blocked with dry milk, and probed with WEDE15 or ATAP2 followed by HRP-coupled goat antimouse and visualization using the Femto detection system (Pierce). In blots showing non–cell-surface proteins loading was assessed using anti–β-actin (Sigma-Aldrich) followed by HRP-coupled goat antirabbit. Optical density of immunoreactive bands was assessed using Scion Image Alpha 4.0.3.2 software (Frederick, MD).

Statistical analysis

Data analysis was performed using the NCSS Statistical & Power Analysis or SigmaStat 3.5 (Systat Software, San Jose, CA) software. A 2-sample 2-tailed homoscedastic *t* test was used to calculate the indicated *P* values.

Results

APC can induce endothelial barrier protective signaling in the presence of thrombin

Enhancement of endothelial barrier integrity is a sensitive readout for PAR1-dependent signaling by exogenous and endogenously generated APC.[11,23] To test if APC-PAR1 signaling still occurs when thrombin is present at concentrations that are expected to lead to rapid cleavage of the available PAR1, we determined whether locally generated APC can still mediate protective effects in the presence of 1 nM thrombin. Neither thrombin alone nor PC zymogen affected permeability of a monolayer of endothelial cells in a dual chamber system after 3 hours of incubation. However, when the cells were coincubated with thrombin and PC, the generated APC significantly enhanced the barrier function (Figure 1A). We next studied the effect of increasing thrombin concentrations on APC's barrier enhancing effects. To only vary the thrombin but not the APC concentration, APC was exogenously added in these experiments, although exogenous APC is less efficient than

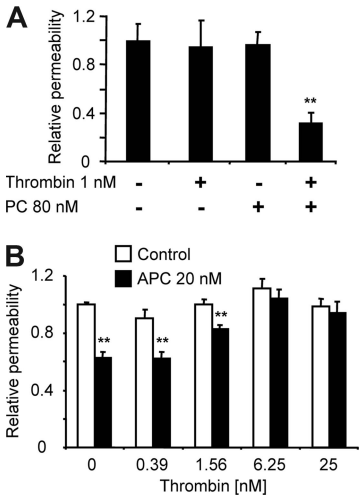

Figure 1. Protective signaling by APC in the presence of thrombin. EA.hy926 cells in a dual-chamber system were incubated for 3 hours with the indicated agonists in the top chamber followed by analysis of permeability. Means plus or minus SEM with n = 5 (A) and 10 (B). $^{**}P < .005$.

endogenously generated APC. Again APC enhanced the barrier integrity despite the presence of up to low-nanomolar thrombin concentrations (Figure 1B). These data indicate that exogenous and endogenously generated APC still mediate barrier protective effects even if thrombin is also present. The possibility that thrombin becomes inhibited or sequestered, allowing APC to induce protective effects through newly externalized PAR1,[25] was ruled out in control experiments that demonstrated that thrombin's proteolytic activity in the cell medium was stable under our experimental conditions (not shown).

Quantification of endogenous PAR1 on the endothelial cell surface by immunoassays

Because it is not possible to directly prove that APC's barrier enhancing effects in the presence of thrombin-PAR1 signaling still depend on PAR1 cleavage, we established assays to assess cleavage of endogenous PAR1 by APC and thrombin alone and in combination. A tagged PAR1 construct has been used to measure PAR1 cleavage in a previous report.[16] However, such transfected PAR1 constructs were later shown to have decreased susceptibility toward cleavage by APC as compared with endogenously expressed PAR1, most likely because of relative unavailability of EPCR.[26] We therefore established a quantitative cell-surface ELISA to analyze how cleavage and/or conformation of endogenous endothelial cell PAR1 are affected. Control experiments using

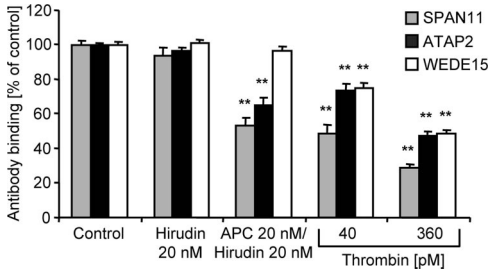

Figure 2. Quantification of cell surface–exposed PAR1. Confluent EA.hy926 cells were incubated for 3 hours with the indicated agonists. Apical expression of PAR1 was analyzed in a cell-surface ELISA using monoclonal anti-PAR1 antibodies SPAN11, ATAP2, and WEDE15. Results are shown relative to control (means ± SEM, n = 9, $^{**}P < .005$).

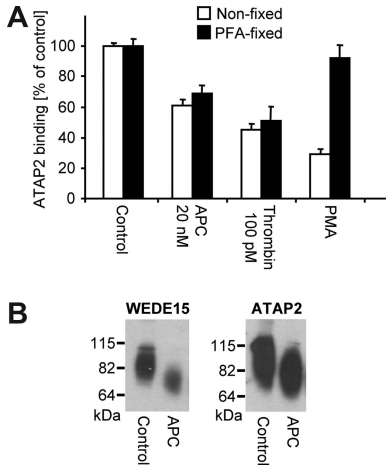

Figure 3. ATAP2 does not detect PAR1's active conformation generated after scissile bond cleavage. (A) ATAP2 binding was quantified by cell-surface ELISA after incubation for 3 hours with the indicated agonists in cells before and after fixation with paraformaldehyde (PFA). PMA indicates phorbol myristate acetate (0.1 μg/mL). Means plus or minus SEM are shown (n = 12). (B) Cells were incubated for 3 hours with control or APC (20 nM) and biotinylated surface proteins were analyzed by Western blotting with WEDE15 or ATAP2 as indicated. A representative experiment of 3 is shown.

immunofluorescence microscopy demonstrated that the anti-PAR1 antibodies under conditions used for the ELISA indeed only bind to cell surface PAR1 and do not detect intracellular pools (Figure S1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Thrombin dose–dependently down-regulated binding of 3 monoclonal antibodies that were raised against the N-terminal exodomain of human PAR1, ie, SPAN11, ATAP2, and WEDE15[20,21] (Figure 2), consistent with the expected cleavage and internalization of PAR1.[27] APC incubation strongly decreased binding of the cleavage-sensitive SPAN11, indicating that APC reduces surface availability of native, uncleaved PAR1. Binding of the non–cleavage-sensitive WEDE15, which binds to an epitope downstream from the Arg41 cleavage site in PAR1's N-terminal exodomain, was not affected by APC, indicating that levels of total surface PAR1 remain constant. These data demonstrate for the first time directly that incubation with APC leads to cleavage of endogenous endothelial cell PAR1. APC down-regulated ATAP2 binding although ATAP2 also binds to an epitope downstream from the Arg41 cleavage site and is regarded as a non–cleavage-sensitive anti-PAR1. This unexpected result indicates that the APC-cleaved PAR1 is detected on the cell surface by WEDE15 but not by ATAP2. ATAP2 recognizes PAR1's Leu44-Arg46 amino acid stretch while WEDE15 binds further downstream at Phe55-Glu60.[20,21] Previous data indicate that APC can only directly cleave PAR1's N-terminal exodomain at Arg41.[28] Incubation with APC might lead to activation of other cellular proteases that in turn might cleave PAR1 at additional sites and cause shedding of the ATAP2 but not the WEDE15 epitope. However, effects of APC on ATAP2 staining were found to be comparable in native cells and cells that have been fixed before agonist incubation (Figure 3A), suggesting that loss of ATAP2 binding is mediated by a conformational change after cleavage. Western blotting of biotinylated cell-surface proteins was used to test whether the ATAP2 epitope can be detected in denatured cleaved PAR1 after APC incubation. The surface biotinylation–based assay does not detect intracellular PAR1, as shown in control experiments (Figure S1B). PAR1 migrated as an elongated immunoreactive band as reported earlier and prolonged incubation with APC led to a cleavage product at the expected smaller size.[28,29] Both WEDE15 and ATAP2 anti-PAR1 similarly detected native and cleaved PAR1 (Figure 3B). The ATAP2 epitope in APC-cleaved PAR1 is thus not shed or degraded and becomes available for ATAP2 binding in denatured PAR1. In conclusion, WEDE15 is a non–cleavage-sensitive antibody and detects total PAR1 in both native (ELISA) and denaturing (Western blotting) conditions. ATAP2 does also bind to cleaved and uncleaved denatured PAR1; however, it is conformation sensitive and does not bind to the cleaved PAR1 in the cell-surface immunoassay.

PAR1 cleavage by APC

Given that ATAP2 is commercially available and sensitive to the conformational change upon cleavage of PAR1 we used this antibody to assess cleavage of endogenously expressed PAR1. Results were confirmed using SPAN11 as indicated below. APC and thrombin down-regulated the binding of ATAP2 in a time dependent manner and 20 nM of APC was similarly efficient as 40 pM thrombin (Figure 4A). A proteolytically inactive recombinant APC variant with an S→A substitution at the active center did not affect ATAP2 binding (Figure 4B). As expected, PAR1 cleavage by APC was completely inhibited in the presence of an antibody against EPCR that blocks APC binding to EPCR (Figure 4C). In contrast, APC significantly down-regulated ATAP2 binding in the presence of nonblocking anti-EPCR, albeit to a lesser extent than in the absence of antibody. A possible explanation for the effect of nonblocking anti-EPCR might be that the antibody leads to clustering and internalization of EPCR during the 3-hour experiment. Down-regulation of ATAP2 binding in response to APC thus requires both APC's proteolytic activity and APC binding to EPCR. For all subsequent experiments analyzing effects of exogenously added APC a concentration of 20 nM was used and agonist

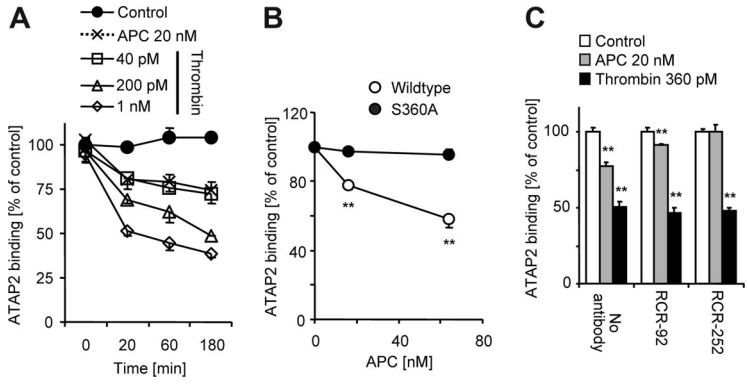

Figure 4. APC down-regulates ATAP2 binding dependent on proteolytic activity and EPCR binding. (A) ATAP2 binding was quantified by ELISA after incubation with the indicated agonists in time course experiments. (B) Cells were incubated for 3 hours with the indicated concentrations of recombinant wild-type APC or proteolytically inactive APC S360A and ATAP2 binding was quantified. (C) Cells were preincubated (10 minutes) in the absence and presence of nonblocking (RCR-92) or blocking (RCR-252) anti-EPCR (25 μg/mL) followed by 3 hours of incubation with the indicated agonists and quantification of ATAP2 binding. Means plus or minus SEM are shown (n = 6 in panels A,C; n = 9 in panel B. $^{**}P < .005$).

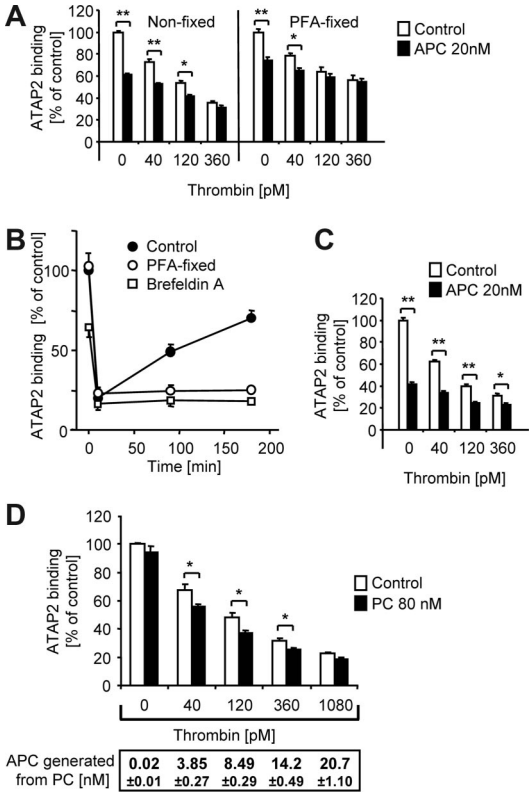

Figure 5. Both exogenous and endogenously generated APC support additional PAR1 cleavage in the presence of thrombin. (A) Nonfixed or PFA-fixed cells were incubated for 3 hours with different concentrations of thrombin in the absence or presence of APC as indicated, followed by analysis of ATAP2 binding. (B) Cells were incubated with 20 nM thrombin for 10 minutes followed by quenching of the protease with 50 nM hirudin. Recovery of ATAP2 binding upon incubation at 37°C over 3 hours is plotted. Where indicated, the cells were PFA-fixed or pretreated with brefeldin A (1 μM, 10 minutes) before the addition of thrombin. A representative experiment (of 3) is shown. (C) Brefeldin A–treated cells were incubated for 3 hours as indicated followed by analysis of ATAP2 binding. (D) Nonfixed cells were incubated with the indicated concentrations of thrombin in the absence or presence of 80 nM PC. After 3 hours the APC concentration in the conditioned medium was determined by chromogenic assay and ATAP2 binding was analyzed. Means plus or minus SEM are shown (n = 9 in panels A,C,D. $*P < .05$, $**P < .005$).

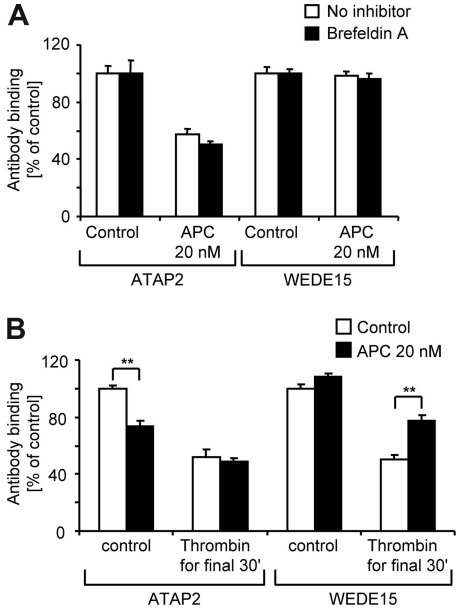

Figure 6. APC cleaved PAR1 remains on the cell surface. (A) Cells were incubated with agonists for 3 hours in the absence or presence of brefeldin A followed by analysis of ATAP2 or WEDE15 binding by surface ELISA as indicated. Means plus or minus SEM are shown (n = 9). (B) Cells were incubated for 3.5 hours with 100 pM hirudin without or with additional 20 nM of APC. For the final 30 minutes of incubation either control or 500 pM of thrombin was added and cell surface–expressed PAR1 was quantified by analysis of ATAP2 and WEDE15 binding. Means plus or minus SEM are shown (n = 9, $**P < .005$ comparing results without and with thrombin for final 30 minutes).

incubation was for 3 hours to obtain significant EPCR-dependent cleavage of PAR1.

APC can mediate additional PAR1 cleavage even if thrombin is also present

When APC was coincubated with thrombin an additional loss of ATAP2 binding was detected (Figure 5A). APC also caused an additional loss of ATAP2 binding in cells that were fixed before agonist incubation consistent with direct effects of APC on PAR1. Endothelial cells have intracellular pools of PAR1 and previous studies have shown that both native and cleaved PAR1 are translocated to the cell surface upon agonist treatment.[20,25] As shown in Figure 5B, the cell surface was repopulated with PAR1 after a high-dose thrombin challenge with similar kinetics in the EA.hy926 cell line as described previously for HUVECs. Brefeldin A, an inhibitor of vesicle trafficking that blocks exocytosis but not internalization of membrane proteins,[30] prevented repopulation of the cell surface with ATAP2 binding sites (Figure 5B). When surface repopulation was blocked with brefeldin A, APC caused a significant additional loss of ATAP2 binding in the presence of up to 360 pM thrombin (Figure 5C). Similar results were obtained using the cleavage-sensitive antibody SPAN11 (not shown). We have previously shown that the endogenous PC activation pathway is mechanistically linked to efficient PAR1-dependent protective signaling.[23] Consistent with these results, when cells were incubated with thrombin in the presence of PC zymogen, ATAP2 binding (and SPAN11 binding; not shown) was further decreased even when only low nM APC was generated (Figure 5D).

APC-cleaved PAR1 is inefficiently removed from the cell surface

Given that EA.hy926 cells can rapidly externalize PAR1 to the cell surface after agonist treatment (Figure 5B), our finding that APC does not affect WEDE15 binding (Figure 2) could be explained by efficient repopulation of the cell surface with cleaved and/or uncleaved PAR1 from intracellular pools. However, APC did not down-regulate WEDE15 staining even in the presence of brefeldin A (Figure 6A), demonstrating that, in contrast to thrombin-cleaved PAR1, APC-cleaved PAR1 is retained on the cell surface. The cleaved PAR1 present on the cell surface after APC incubation was still retained after a subsequent incubation with high-dose thrombin (Figure 6B).

These results raise the possibility that APC-cleaved PAR1 might accumulate on the cell surface even in the presence of thrombin. When ATAP2 (detects only uncleaved PAR1) and WEDE15 (detects both cleaved and uncleaved PAR1) binding was analyzed in parallel experiments after incubation with increasing concentrations of thrombin alone, staining with both antibodies was almost identical (Figure 7A). This finding indicates that no detectable cleaved PAR1 is retained on the cell surface upon thrombin incubation. If the cells were coincubated with APC, ATAP2 binding was further decreased, consistent with additional cleavage of PAR1 by APC in the presence of thrombin. In contrast, coincubation with APC led to enhanced WEDE15 binding, demonstrating that total

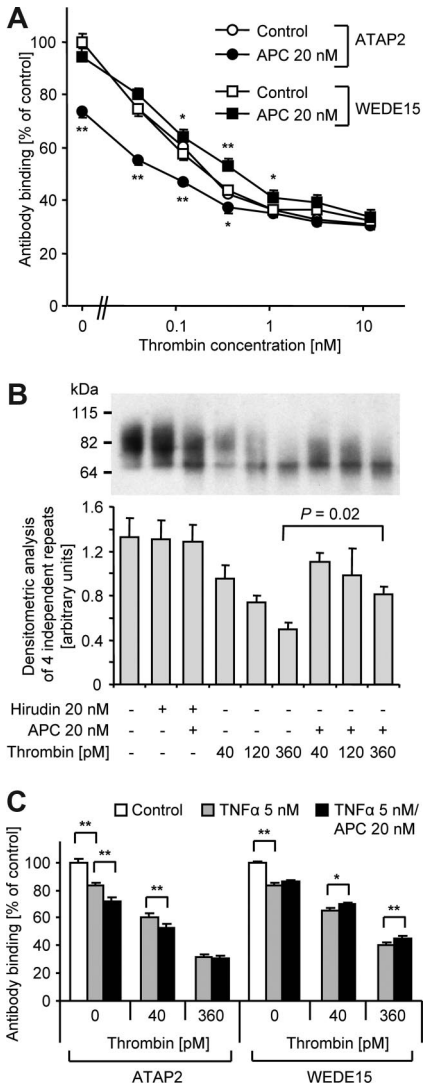

Figure 7. APC-cleaved PAR1 is retained on the cell surface even in the presence of thrombin. (A) Nonfixed cells were incubated for 3 hours with the indicated agonists and cell surface–expressed PAR1 was quantified by analysis of ATAP2 and WEDE15 binding. (B) After incubation with the indicated agonists for 3 hours, surface proteins were biotinylated and isolated with streptavidin agarose. PAR1 was detected by Western blotting using anti-PAR1 WEDE15. A representative experiment is shown in the top panel. Optical density of immunoreactive bands was measured in 3 independent experiments and means plus or minus SEM are shown in the bottom panel. Coincubation with APC led to detection of more surface PAR1 at all thrombin concentrations, a finding that was borderline significant at lower thrombin concentrations but significant at 360 pM thrombin. (C) As indicated, cells were induced with TNFα for 2 hours and thrombin and/or APC added for an additional 3 hours followed by quantification of ATAP2 and WEDE15 binding. Means plus or minus SEM are shown in panels A,C (n = 15 in panel A and 7 in panel C, $*P < .05$, $**P < .005$, comparing results without and with APC in panel A).

(cleaved and uncleaved) PAR1 is increased. Because the differential between WEDE15 and ATAP2 staining corresponds to the amount of cleaved PAR1 these findings show that treatment with APC results in the retention of cleaved PAR1 on the cell surface in the absence as well as in the presence of up to 1 nM thrombin. Analysis of biotinylated cell-surface proteins by Western blotting confirmed that coincubation with APC reproducibly led to higher levels of PAR1 on the cell surface compared with thrombin alone (Figure 7B). To test whether similar results are also obtained in inflammatory cytokine-perturbed endothelial cells, we analyzed cleavage and surface retention of PAR1 in response to incubation with APC and thrombin in cells that were pretreated with a high concentration of tumor necrosis factor-α (TNFα; Figure 7C). TNFα reduced the amount of surface-exposed native PAR1 and PAR1 cleavage by APC was found to be slightly less efficient in TNFα-induced cells most likely because of decreased EPCR expression. However, APC still supported significant PAR1 cleavage in both the absence and presence of thrombin and the APC-cleaved PAR1 was surface retained, entirely consistent with our results in quiescent cells. Although the differences were relatively small in TNFα-pretreated cells, the data show that APC leads to a unique population of cleaved PAR1 on the cell surface that might mediate APC's beneficial effects in systemic inflammation.

Discussion

In sepsis the efficient PAR1 activator thrombin is generated, and therefore the question how any beneficial effects of APC can be PAR1 mediated is at the root of the current controversy. So far it was difficult to explain how APC can mediate any effects through PAR1 because thrombin is much more efficient in cleaving this receptor. Our data directly demonstrate for the first time that APC can cleave endogenous PAR1 on the surface of human endothelial cells, corroborating conclusions from studies using overexpressed tagged PAR1 constructs[16] or cleavage blocking antibodies against PAR1.[6] Exogenous and endogenously generated APC can mediate both endothelial barrier protective signaling and additional cleavage of endogenous PAR1 on the endothelial cell surface even in the presence of up to 1 nM of thrombin. The absence of detectable additional cleavage of epitope tagged PAR1 by generated APC in a previous study[16] could be explained by preferential cleavage of the overexpressed receptor by thrombin that binds directly to PAR1 and does not require a coreceptor.[26] Our finding that APC has no detectable additional effect on PAR1 cleavage in the presence of high thrombin concentrations (> 1 nM) argues that any distinct PAR1 population that can only be cleaved by APC but not by thrombin would have to be very small. Consistent with the findings by Bae et al[26] we propose that there is a PAR1 population that is colocalized with EPCR and that is cleaved by APC. Even though thrombin can also cleave this PAR1 population, cleavage by APC is efficient enough to lead to significant additional cleavage at up to high-picomolar thrombin concentrations.

Whereas thrombin-cleaved PAR1 is rapidly internalized and degraded,[27] our data show that APC-activated PAR1 remains on the cell surface and accumulates upon prolonged incubation even when thrombin is present. It has been recently established that downstream G-protein coupling of PAR1 differs for specific agonists.[31] Therefore, it is conceivable that even though thrombin and APC activate PAR1 by cleaving the same scissile bond[28] differences in the interaction of the tethered ligand with PAR1 could lead to the activation of distinct downstream signaling pathways and to unique biologic responses, including distinct trafficking of thrombin- and APC-cleaved PAR1. Such differences might also explain the finding that thrombin and APC can have distinct effects on gene expression through PAR1 signaling in cytokine-perturbed endothelial cells.[8] Our previous results demonstrate that low (~40 pM) but not high thrombin concentrations can induce endothelial barrier protection similar to APC,[11] suggesting that differences in the PAR1 cleavage rate translate into distinct downstream signaling. Our present results that APC can mediate protective effects in the presence of thrombin concentrations much higher than 40 pM indicate that differences in the cleavage rate alone cannot explain

all the findings. One possibility is that APC binding to EPCR leads to distinct signaling of the colocalized PAR1.[32] Although extensive research will be required to elucidate molecular details of the downstream signaling pathways and to establish that the surface-retained PAR1 indeed mediates protective APC effects in the presence of thrombin, our results provide conceptually novel insight into the paradoxical condition that the 2 key coagulation proteases thrombin and APC, linked by a negative feedback loop, can mediate opposite effects on endothelial biology through the same receptor PAR1.

Our finding that cellular trafficking of thrombin- and APC-cleaved PAR1 is distinct suggests how receptor signaling by a very inefficient protease can be relevant in the presence of the much stronger agonist thrombin. Because of the irreversibility of proteolytic activation PAR1 signaling must be regulated through mechanisms such as receptor trafficking.[27] Although the rate of thrombin-PAR1 cleavage at any given point in time might be much higher than the rate of APC-PAR1 cleavage, the thrombin-cleaved receptor is rapidly internalized and degraded whereas the APC-cleaved receptor accumulates on the surface and can potentially mediate relevant signaling in the presence of thrombin. This illustrates how the efficiency of induction of a specific biologic response does not necessarily correlate with efficiency of cleavage. This concept cannot only explain our finding that APC enhances endothelial barrier integrity in the presence of thrombin, but it might help to clarify the complex roles of PAR1 in vivo. PAR1 deficiency did not affect survival in mouse models of endotoxemia,[33,34] even though PAR1 has well-established proinflammatory effects in other models, such as glomerulonephritis, inflammatory bowel disease, or ischemia-reperfusion injury.[35-37] This argues that any detrimental effects of PAR1 deficiency in systemic inflammation might be offset by the absence of proinflammatory PAR1 signaling. Very recent results indeed support the conclusion that PAR1 has such dual roles during different stages of the inflammatory response in mouse models of severe sepsis.[38,39] These findings suggest that PAR1 mediates protective signaling by the PC pathway in vivo under conditions where thrombin is also present. Our in vitro data suggest that relevant protective PAR1 signaling by the PC pathway might be possible in the presence of up to high-picomolar or low-nanomolar thrombin. PAR1 is activated with half maximal efficiency by a concentration of only approximately 50 pM thrombin[14] and thrombin activity in vivo is rigorously controlled to prevent excessive platelet activation/fibrin formation. Although it is not known what the thrombin concentration in the endothelial cell microenvironment under conditions of inflammatory stress might be, data from Dr Coughlin's laboratory elegantly demonstrate that thrombin likely operates close to threshold levels: In murine platelets PAR4 cleavage is required for thrombin signaling and PAR3 acts as a nonsignaling cofactor that recruits thrombin and decreases the concentration required for half maximal signaling between 6- and 15-fold.[40] However, PAR3- and PAR4-deficient mice showed similar degrees of protection in thrombosis models, indicating that an approximately 10-fold decrease in platelet responsiveness to thrombin has the same effect as complete unresponsiveness.[41,42] Based on these findings, we expect that the range of thrombin concentrations where we obtained protective signaling and surface retention of APC-cleaved PAR1 is of physiologic relevance.

In conclusion, our results help explain how using a single receptor cell can sense proteolytic activity of thrombin and APC independently, and they support the concept that PAR1-dependent signaling might contribute to protective effects of APC in sepsis.

Acknowledgments

We thank Drs J. Griffin, K. Fukudome, and C. Edgell for valuable reagents.

This work was supported by National Institutes of Health grant HL 73 318 (M.R.), grants from the Swiss National Science Foundation PBBBE-108544, the Stiefel-Zangger Foundation of the University of Zurich, and the Theodor and Ida Herzog-Egli-Stiftung (R.A.S.), and an Austrian fellowship, Erwin Schrödinger-Auslandsstipendium J2413-B13 (C.F.).

Authorship

Contribution: R.A.S., C.F., and M.R. designed and performed research and analyzed data; R.A.S and M.R. wrote the manuscript; and L.F.B contributed monoclonal antibodies against PAR1.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Matthias Riewald, MD, Scripps Research Institute, Department of Immunology, SP30-3040, 10550 North Torrey Pines Rd, La Jolla, CA 92037; e-mail: riewald@scripps.edu.

References

1. Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med. 2001; 344:699-709.
2. Esmon CT. The protein C pathway. Chest. 2003; 124:26S-32S.
3. Warren BL, Eid A, Singer P, et al. Caring for the critically ill patient. High-dose antithrombin III in severe sepsis: a randomized controlled trial. JAMA. 2001;286:1869-1878.
4. Abraham E, Reinhart K, Opal S, et al. Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial. JAMA. 2003;290:238-247.
5. Taylor FB, Jr., Chang AC, Peer GT, et al. DEGR-factor Xa blocks disseminated intravascular coagulation initiated by Escherichia coli without preventing shock or organ damage. Blood. 1991;78: 364-368.
6. Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science. 2002;296:1880-1882.
7. Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. Blood. 2007;109: 3161-3172.
8. Riewald M, Ruf W. Protease-activated receptor-1 signaling by activated protein C in cytokine-perturbed endothelial cells is distinct from thrombin signaling. J Biol Chem. 2005;280:19808-19814.
9. Cheng T, Liu D, Griffin JH, et al. Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. Nat Med. 2003;9:338-342.
10. Mosnier LO, Griffin JH. Inhibition of staurosporine-induced apoptosis of endothelial cells by activated protein C requires protease-activated receptor-1 and endothelial cell protein C receptor. Biochem J. 2003;373:65-70.
11. Feistritzer C, Riewald M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. Blood. 2005;105:3178-3184.
12. Guo H, Liu D, Gelbard H, et al. Activated protein C prevents neuronal apoptosis via protease activated receptors 1 and 3. Neuron. 2004;41:563-572.
13. Cheng T, Petraglia AL, Li Z, et al. Activated protein C inhibits tissue plasminogen activator-induced brain hemorrhage. Nat Med. 2006;12: 1278-1285.
14. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell. 1991;64:1057-1068.
15. Vu TK, Wheaton VI, Hung DT, Charo I, Coughlin SR. Domains specifying thrombin-receptor interaction. Nature. 1991;353:674-677.
16. Ludeman MJ, Kataoka H, Srinivasan Y, Esmon NL, Esmon CT, Coughlin SR. PAR1 cleavage and signaling in response to activated protein C and thrombin. J Biol Chem. 2005;280:13122-13128.
17. Esmon CT. Is APC activation of endothelial cell PAR1 important in severe sepsis?: No. J Thromb Haemost. 2005;3:1910-1911.

18. Riewald M, Ruf W. Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. Proc Natl Acad Sci U S A. 2001;98:7742-7747.
19. Fernandez JA, Xu X, Liu D, Zlokovic BV, Griffin JH. Recombinant murine-activated protein C is neuroprotective in a murine ischemic stroke model. Blood Cells Mol Dis. 2003;30:271-276.
20. Hoxie JA, Ahuja M, Belmonte E, Pizarro S, Parton R, Brass LF. Internalization and recycling of activated thrombin receptors. J Biol Chem. 1993;268:13756-13763.
21. Brass LF, Pizarro S, Ahuja M, et al. Changes in the structure and function of the human thrombin receptor during receptor activation, internalization, and recycling. J Biol Chem. 1994;269:2943-2952.
22. Ye X, Fukudome K, Tsuneyoshi N, et al. The endothelial cell protein C receptor (EPCR) functions as a primary receptor for protein C activation on endothelial cells in arteries, veins, and capillaries. Biochem Biophys Res Commun. 1999;259:671-677.
23. Feistritzer C, Schuepbach RA, Mosnier LO, et al. Protective signaling by activated protein C is mechanistically linked to protein C activation on endothelial cells. J Biol Chem. 2006;281:20077-20084.
24. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci U S A. 1983;80:3734-3737.
25. Woolkalis MJ, DeMelfi TM, Jr., Blanchard N, Hoxie JA, Brass LF. Regulation of thrombin receptors on human umbilical vein endothelial cells. J Biol Chem. 1995;270:9868-9875.
26. Bae JS, Yang L, Rezaie AR. Receptors of the protein C activation and activated protein C signaling pathways are colocalized in lipid rafts of endothelial cells. Proc Natl Acad Sci U S A. 2007;104:2867-2872.
27. Shapiro MJ, Coughlin SR. Separate signals for agonist-independent and agonist-triggered trafficking of protease-activated receptor 1. J Biol Chem. 1998;273:29009-29014.
28. Kuliopulos A, Covic L, Seeley SK, Sheridan PJ, Helin J, Costello CE. Plasmin desensitization of the PAR1 thrombin receptor: kinetics, sites of truncation, and implications for thrombolytic therapy. Biochemistry. 1999;38:4572-4585.
29. Vouret-Craviari V, Grall D, Chambard JC, Rasmussen UB, Pouyssegur J, Van Obberghen-Schilling E. Post-translational and activation-dependent modifications of the G protein-coupled thrombin receptor. J Biol Chem. 1995;270:8367-8372.
30. Klausner RD, Donaldson JG, Lippincott-Schwartz J. Brefeldin A: insights into the control of membrane traffic and organelle structure. J Cell Biol. 1992;116:1071-1080.
31. McLaughlin JN, Shen L, Holinstat M, Brooks JD, Dibenedetto E, Hamm HE. Functional selectivity of G protein signaling by agonist peptides and thrombin for the protease-activated receptor-1. J Biol Chem. 2005;280:25048-25059.
32. Bae JS, Yang L, Manithody C, Rezaie AR. The ligand occupancy of endothelial protein C receptor switches the PAR-1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. Blood. 2007;110:3909-3916.
33. Pawlinski R, Pedersen B, Schabbauer G, et al. Role of tissue factor and protease-activated receptors in a mouse model of endotoxemia. Blood. 2004;103:1342-1347.
34. Camerer E, Cornelissen I, Kataoka H, Duong DN, Zheng YW, Coughlin SR. Roles of protease-activated receptors in a mouse model of endotoxemia. Blood. 2006;107:3912-3921.
35. Cunningham MA, Rondeau E, Chen X, Coughlin SR, Holdsworth SR, Tipping PG. Protease-activated receptor 1 mediates thrombin-dependent, cell-mediated renal inflammation in crescentic glomerulonephritis. J Exp Med. 2000;191:455-462.
36. Vergnolle N, Cellars L, Mencarelli A, et al. A role for proteinase-activated receptor-1 in inflammatory bowel diseases. J Clin Invest. 2004;114:1444-1456.
37. Sevastos J, Kennedy SE, Davis DR, et al. Tissue factor deficiency and PAR-1 deficiency are protective against renal ischemia reperfusion injury. Blood. 2007;109:577-583.
38. Kerschen EJ, Fernandez JA, Cooley BC, et al. Endotoxemia and sepsis mortality reduction by non-anticoagulant activated protein C. J Exp Med. 2007;204:2439-2448.
39. Kaneider NC, Leger AJ, Agarwal A, et al. 'Role reversal' for the receptor PAR1 in sepsis-induced vascular damage. Nat Immunol. 2007;8:1303-1312.
40. Nakanishi-Matsui M, Zheng YW, Sulciner DJ, Weiss EJ, Ludeman MJ, Coughlin SR. PAR3 is a cofactor for PAR4 activation by thrombin. Nature. 2000;404:609-613.
41. Sambrano GR, Weiss EJ, Zheng YW, Huang W, Coughlin SR. Role of thrombin signalling in platelets in haemostasis and thrombosis. Nature. 2001;413:74-78.
42. Weiss EJ, Hamilton JR, Lease KE, Coughlin SR. Protection against thrombosis in mice lacking PAR3. Blood. 2002;100:3240-3244.

Published in final edited form as:
Thromb Haemost. 2009 April ; 101(4): 724–733.

Protection of Vascular Barrier Integrity by Activated Protein C in Murine Models Depends on Protease-Activated Receptor-1

Reto A. Schuepbach[1,*], **Clemens Feistritzer**[1,*], **José A. Fernández**[2], **John H. Griffin**[2], and **Matthias Riewald**[1]

1*Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, USA.*

2*Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, USA.*

Abstract

Protease activated receptor-1 (PAR1) mediates barrier protective signaling of activated protein C (APC) in human endothelial cells *in vitro* and may contribute to APC's beneficial effects in patients with severe sepsis. Mouse models are of key importance for translational research but species differences may limit conclusions for the human system. We analyzed whether mouse APC can cleave, activate and induce signaling through murine PAR1 and tested in newly established mouse models if long term infusion of APC prevents from vascular leakage. Cell surface immunoassays demonstrated efficient cleavage of endogenous murine endothelial PAR1 by either murine or human APC. Pharmacological concentrations of APC of either species had powerful barrier protective effects on cultured murine endothelial cells that required PAR1 cleavage. Vascular endothelial growth factor-mediated hyperpermeability in the skin was reduced by either endogenously generated as well as directly infused recombinant mouse APC in wild type mice. However APC did not significantly alter the vascular barrier function in PAR1-deficient mice. In endotoxin challenged mice, infused APC significantly prevented from pulmonary fluid accumulation in the wild type but not in mice lacking PAR1. Our results directly show that murine APC cleaves and signals through PAR1 in mouse endothelial cells. APC reduces vascular permeability in mouse models and PAR1 plays a major role in mediating these effects. Our *in vitro* and *in vivo* data support the paradigm that PAR1 contributes to protective effects of APC on vascular barrier integrity in sepsis.

Keywords

activated protein C; endothelial cells; protease-activated receptor-1; sepsis, vascular barrier

Infusion of recombinant human activated protein C (APC) can reduce mortality in patients with severe sepsis (1). APC downregulates the generation of thrombin, the key procoagulant enzyme of the blood coagulation system (2,3) but these anticoagulant effects of APC are unlikely to explain its benefit in septic patients because other anticoagulants improved the sepsis related coagulopathy but failed to improve survival of septic patients (4,5). Consistent with this conclusion, recent results show that a non-anticoagulant variant of APC can protect mice in an endotoxemia model similarly well as wildtype APC (6).

APC can mediate a number of potentially protective effects in human endothelial cells that include stabilization of barrier integrity, anti-apoptotic effects, and downregulation of adhesion

Corresponding author: Matthias Riewald, M.D. The Scripps Research Institute, SP30-3040 Department of Immunology and Microbial Science 10550 North Torrey Pines Rd La Jolla, CA 92037 Tel: 858 784 8226 Fax: 858 784 7276 Email: E-mail: riewald@scripps.edu.
*These authors contributed equally to this work.

receptors (7). These cytoprotective activities of APC require binding to the endothelial protein C receptor (EPCR) and are at least in part mediated by signaling through the thrombin receptor protease activated receptor-1 (PAR1), a G protein-coupled receptor that is activated by proteolytic cleavage of its N-terminal exodomain (8,9). PAR1 has been implicated in protective *in vivo* effects of APC in mouse models, including increased survival of APC treated mice in endotoxemia (6). Both human and mouse APC has been used in mouse models but it is unknown whether significant species differences exist in APC signaling. PAR1 is known to play distinct roles in different species, e.g. PAR1 mediates platelet activation in humans but not in mice (8). So far, it has never been tested directly whether human or mouse APC can cleave and activate PAR1 on mouse endothelial cells. Indirect evidence that in the mouse APC cleaves and activates PAR1 was drawn from in vivo studies (10) using PAR1 blocking antibodies. However, recent reports show that APC binding to EPCR can mediate protective PAR1-dependent signaling even if this receptor gets cleaved by another protease such as thrombin (11). Since mouse models are widely used in translational research to understand how APC therapy improves survival of septic patients more direct insights in whether the APC-PAR1 pathway is conserved between mouse and humans is important.

Dysfunction of the vascular barrier is a key event in the pathogenesis of sepsis and plays an important role in the development of organ dysfunction, such as the lung injury-triggered development of acute respiratory distress syndrome. Previous studies have shown that APC can attenuate acute lung injury (12,13). Enhancement of endothelial barrier integrity is a highly sensitive downstream effect of APC-PAR1 signaling in cultured human endothelial cells that requires crossactivation of sphingosine 1-phosphate (S1P) receptors (14,15). Given that S1P can reduce the vascular leak in animal models of acute lung injury (16,17), it is tempting to speculate that vascular barrier protection may contribute to beneficial effects of APC treatment in sepsis.

Here we show that in cultured mouse endothelial cell lines APC directly cleaves and activates endogenous PAR1 which leads to reduced permeability of an endothelial cell monolayer. Infused or *in vivo* generated APC significantly enhanced vascular barrier integrity in wildtype mice but not in PAR1-deficient mice. The findings support the concept that PAR1-dependent protection of vascular barrier integrity contributes to beneficial effects of APC in sepsis.

METHODS

Reagents and Antibodies

Human plasma-derived APC and PAR1 agonist peptide were as described previously (9,14, 18-20). Recombinant mouse APC was made as described (21). Human WE-thrombin was kindly provided by Dr. Di Cera (Washington University). All *in vitro* experiments involving stimulation with APC included hirudin (Calbiochem, La Jolla, CA). Mouse thrombin was from Haematologic Technologies (Essex Junction, VT). The S-19 polyclonal goat anti-mouse PAR1 (Lot# L1205) and its commercial blocking peptide (RSFFLRNPSENTFELVPLGDE) were from Santa Cruz (Santa Cruz, CA). Additional peptides corresponding to the N-terminus of mature mouse PAR1 were custom synthesized (CHI Scientific Inc., MA USA) and used for mapping the PAR1 epitope recognized by the S-19 anti-PAR1 antibody. Mouse plasma IL-6 was quantified by DuoSet ELISA (R&D Systems, Minneapolis, MN) and thrombin antithrombin complexes by the ELISA for TAT complexes (Enzyme Research Laboratories, South Bend, IN) following the protocols of the manufactures. Mouse APC plasma levels were determined as described (22). In brief, mouse blood was collected into vials containing 0.1 M citrate and 10 mM benzamidine (final concentrations). After centrifugation, the plasma samples were loaded onto wells precoated with monoclonal anti-mouse PC AMGDPC1587 (kindly provided by Dr. Esmon; Oklahoma Health Sciences Center), incubated for 2 h, and extensively washed with Tris buffer containing 0.05% Tween 20. Serial dilutions of recombinant mouse

APC were used to prepare a standard curve. Amidolytic activity of pulled down proteins was quantified by Spectrozyme PCa (#336, American Diagnostica, Stamford, CT).

Cell Culture

Transduced mouse endothelial cell lines MS1 and b.End3 were from the American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's Modified Eagle Media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Results obtained with both mouse cell lines were similar throughout all *in vitro* assays except for permeability assays where only the b.End3 sufficiently attached to the microporous membrane. Plasmid transfection was performed in human EA.hy926 cells (23). Cells were plated in tissue culture medium together with plasmids and Lipofectamine™2000 (Invitrogen, Carlsbad, CA) which was replaced with growth medium 6 h after transfection and the cells were used for experiments two days later. The plasmids contained the full length cDNA coding for mouse PAR1 in the pcDNA3.1/Zeo(+) vector (Invitrogen, Carlsbad, CA). Sequence analysis confirmed the consistency with gene bank entry #W20934. Full length human PAR4 in the same vector was used as a control.

Permeability Assay and Surface Immunoassay

The macromolecular monolayer permeability was assessed as described (14). Briefly b.End3 endothelial cells were grown to subconfluence for two days on polycarbonate membrane transwells (Costar, 3 μM pore size) serum starved for 30 min in absence or presence of the PAR1 blocking antibody S19. After a 3 h incubation with agonists all media were removed and DMEM containing 0.67 mg/mL of Evans Blue and 4% bovine serum albumin was added to the transwell compartment above the microporous membrane. Changes in endothelial permeability were quantified by measuring the increase in absorbance at 650 nm of the medium (DMEM containing 0.4% bovine serum albumin) placed into the bottom well. Cell surface PAR1 was quantified by an immunoassay as described (20) except that S-19 goat anti-mouse PAR1 at 2 μg/ml and a peroxidase-coupled rabbit anti-goat IgG (Calbiochem) were used. Modifications of this assay were used in order to map (the) binding site(s) of S-19 on mouse PAR1. On one hand, it was tested whether an excess (100 nM) of peptide can compete with endogenously expressed mouse PAR1 on MS1 cells for S-19 binding. On the other hand, it was tested whether the immobilized peptides are able to bind to S-19.

Immunoprecipitation, and Western Blotting

Cell surface proteins were biotinylated as described (20). PAR1 was immunoprecipitated with S-19 (0.5 μg/ml) and protein G-agarose (Calbiochem). Following extensive washing, eluted proteins were separated by SDS-PAGE, transferred to Immobilon-P-membranes (Millipore, Bedford, MA) and biotinylated proteins were visualized with Streptavidin-HRP (Zymed, San Francisco, CA) and the Femto detection system (Pierce). Western blotting for mitogen-activated protein kinase phosphorylation was as reported (18). Stripped membranes were re-probed with anti-β-actin (Sigma) and analyzed as loading controls.

Mouse Models for Vascular Barrier Integrity

All studies were approved by The Scripps Research Institute Animal Care and Use Committee and comply with National Institutes of Health guidelines. Wildtype C57BL/6 and PAR1-deficient mice, backcrossed for >10 generations into the C57BL/6 background, were as described (24) and bred in-house. Jugular vein catheters were surgically implanted in age-matched 8 to 10 week old male mice 4-7 days before experiments. Implanted mice were free to move and without any signs of distress and systemic markers for inflammation (IL-6) and coagulation (TAT complexes). A microprocessor controlled syringe pump infused 300 μL per hour of vehicle (physiological saline) either alone or containing WE (200 μg/kg/h) or

recombinant mouse APC (120 μg/kg/h). Up to 16 mice were infused simultaneously. LPS (E. coli serotype O111:B4; Sigma, St. Louis, MO) injected mice were sacrificed at the end of the infusion. To quantify vascular leakage in the skin, intravascular albumin was labeled with Evans blue (1 mg in 100 μl intravenous). Recombinant murine vascular endothelial growth factor (VEGF165; Peprotech, Rocky Hill, NJ) was intradermally injected (25 μl; 1 μg/ml) into the preshaved abdominal skin and the injection sites were removed by dermal punch biopsies (diameter 6 mm) 30 min later after the animal was sacrificed. The skin samples were incubated in 200 μl of formamide at 56°C for 24 h and the extracted Evans blue content was quantified by measuring the OD at 620 nm. Results from six samples of each animal were averaged and used as a single data point for further data analysis. In endotoxin (LPS) challenged mice (20 mg/kg; *E.coli* serotype O111:B4: Sigma, St. Louis, MO) pulmonary edema was quantified by measuring the wet to dry weight ratio of the left lung (weight obtained immediately after extirpation divided by the weight assessed after 4 days of drying at 60°C). For histological analysis the cardiac lung lobe was formaldehyde fixed, paraffin embedded and 10 μm microsections stained with Hematoxylin Eosin were analyzed by light microscopy (Olympus BX60, Olympus America INC, San Diego, CA).

Statistical Analysis

Data analysis was performed using the NCSS Statistical & Power Analysis Software. A two-sample two-tailed homoscedastic t-test was used to compare groups of two, parametric ANOVA was used to compare equally powered groups of more than two.

RESULTS

APC Induces Barrier Function in Mouse Endothelial Cells

In a first series of experiments we analyzed effects of APC on endothelial barrier integrity in a dual chamber system. Mouse APC enhanced the barrier function in a dose dependent manner in a mouse brain endothelial cell line (Fig. 1). Mouse APC was slightly more efficient than human APC in this assay. The anti human PAR1 antibody H-111 (Santa Cruz) has been previously reported to block cleavage and was used to establish that APC-PAR1 signaling is required for anti apoptotic effects of APC in mouse neuronal cells (10). Unfortunately, recent lots of this polyclonal antibody did not bind to PAR1 in cultured mouse endothelial cell lines (data not shown), likely due to a change in the properties of this polyclonal antibody raised against the N-terminus of human PAR1. Thus, novel tools had to be established in order to test whether APC can directly cleave PAR1 and thereby enhance the endothelial barrier function in mouse cells.

APC Cleaves Mouse PAR1 and the APC-cleaved Receptor is Retained on the Cell Surface

To test whether APC directly cleaves mouse PAR1 we established an immunoassay to quantify binding of anti-PAR1 to the endothelial cell surface. Transfection studies overexpressing mouse PAR1 in the human endothelial cell line EA.hy926 established specificity of the S-19 polyclonal goat anti-mouse PAR1 (Fig. 2A). Mouse thrombin efficiently removed anti-PAR1 epitopes from cultured mouse endothelial cells whereas mouse APC had no effect (Fig. 2B). These results either suggest that APC cannot cleave mouse PAR1 or that APC-cleaved receptors are not efficiently removed from the cell surface, as described recently for human PAR1 (20). To clarify if cleavage or internalization of mouse PAR1 explains the reduced binding of S-19 in thrombin treated cells we mapped the S-19 epitope(s) (Fig. 2C). The commercially available S-19 blocking peptide (blocked S-19 — used throughout this study), was sequenced and found to contain 20 amino acids beginning with R41 at the scissile bond that leads to receptor activation. This peptide as well as the shorter, custom synthesized peptide NE-15 (amino acids 47 to 61) competes with S-19 binding to endothelial expressed PAR1 and support S-19 binding to the immobilized peptide. In contrast neither the peptide corresponding

to the full length of the mouse PAR1 upstream of the scissile bond (amino acid 22 to 41) nor any other of the custom made peptides bound to S-19 in both assays. These data show that S-19 goat anti-mouse PAR1 antibody is not expected to be cleavage sensitive and that it specifically binds to an epitope containing V56 and P57. Biotinylated cell surface exposed receptors were analyzed by SDS-PAGE to assess agonist dependent cleavage. Densitometric analysis of immunoreactive bands confirmed that the total amount of mouse PAR1 on the cell surface is neither affected by mouse nor by human APC but significantly reduced upon incubation with mouse thrombin (Fig. 2D), consistent with the findings from the surface immunoassay. Importantly, both thrombin and APC led to the appearance of a smaller cleavage product with an average apparent size of approximately 60 kDa. In order to estimate cleavage efficiency we determined the proportion of immunoreactive products above and below PAR1's native average size of 82 kDa. This proportion was significantly reduced not only upon incubation with thrombin but also after APC incubation, indicating that APC does cleave mouse PAR1. As found previously for human PAR1 (20), APC-cleaved mouse PAR1 was surface retained even after a short incubation with high dose thrombin (Fig. 2E) and when cells were coincubated with thrombin and APC or zymogen protein C (not shown). Taken together, these data show for the first time directly, that mouse PAR1 can in fact be cleaved by APC and APC-cleaved receptors are retained on the cell surface similar as described for human PAR1.

APC Signaling and Barrier Protection in Mouse Endothelial Cells Require PAR1 Cleavage

To test the hypothesis that PAR1 cleavage is indeed necessary for APC's effects on the barrier function of cultured mouse endothelial cells, we screened several antibodies raised against PAR1 for their ability to block PAR1 cleavage-mediated signaling. Only S-19 significantly reduced thrombin- but not PAR1 agonist peptide-induced phosphorylation of Erk1/2 in mouse endothelial cells (Fig. 3A). As expected, preincubation of S-19 with its immunizing peptide rendered the antibody inefficient and ruled out non specific effects of S-19. Mouse APC but not human APC induced detectable Erk1/2 phosphorylation in our assay conditions (Fig. 3B), consistent with less efficient signaling of human APC in mouse cells. Preincubation with S-19 blocked Erk1/2 phosphorylation in response to mouse APC. Most importantly, the barrier enhancing effect of mouse APC in cultured mouse endothelial cells was found to be blocked in the presence of anti PAR1 S-19 (Fig. 3C), indicating that PAR1 cleavage is required for the induction of endothelial barrier function by APC in cultured mouse endothelial cells.

Vascular Barrier Protection by APC in the Skin Requires PAR1

APC-PAR1 has powerful endothelial barrier protective effects *in vitro* but relatively high concentrations of APC (>2.5 nM) were required (Fig. 1). Thus, we first attempted to test whether APC similarly affects the vascular barrier *in vivo*. Based upon our *in vitro* studies we expected that a prolonged exposition for several hours to APC may be necessary and that endogenously generated APC may be more efficient compared to exogenous APC (19). Efficient endothelial generation of APC was reported from baboons infused with mutated, anticoagulant thrombin (WE) and WE has a much longer half life in the circulation than APC (25,26). Human WE efficiently activated mouse protein C on cultured endothelial cells (Fig. 4A) and it was about 500 times less efficient in cleaving mouse PAR1 if compared to non mutated mouse thrombin (Fig. 4B). Bolus injections of WE in mice resulted in efficient and sustained *in vivo* generation of APC (Fig. 4C) which was comparable to reports from baboons (26). A continuous infusion with 200 μg/kg/h of WE (without bolus) yielded plasma levels of around 20 ng/ml, comparable to levels achieved in septic patients treated with recombinant APC (Fig. 4D).

To directly test whether exogenous and endogenously generated APC can protect vascular barrier integrity, we analyzed vascular endothelial growth factor (VEGF)-mediated hyperpermeability in a skin model derived from the classic Miles assay (Fig. 5A). The dermal

vascular bed is known to highly express EPCR as well as PAR1 and is therefore expected to support local APC generation and APC-PAR1 signaling. Evans blue was used to label intravascular albumin and control experiments demonstrated that the results indeed reflect time-dependent extravasation in response to increased vascular permeability (not shown). Infusion with WE at a dose that leads to plasma APC levels comparable to APC-treated patients had a highly significant protective effect against VEGF-induced hyperpermeability (Fig. 5B). Direct infusion of mouse APC at a dose (120 μg/kg/h) comparable to the one licensed for treating septic patients also had a significant vascular barrier protective effect. To test if effects of APC depend on PAR1 a set of wildtype and PAR1 deficient mice were parallel infused with either saline alone or APC. Only in mice expressing PAR1 APC had significant effects (Fig. 5C). These results demonstrate that APC can enhance vascular barrier integrity *in vivo* and that PAR1 plays a major role mediating this effect.

PAR1-dependent Protection from Pulmonary Edema by APC in an Endotoxemia Model

To test if APC has similar vascular barrier protective effects in systemic inflammation we assessed pulmonary edema formation at an early time point in an endotoxemia model (Fig. 6A). Intraperitoneal injection of a lethal dose of lipopolysaccharide (LPS) led to a strong increase of pulmonary fluid content after 5 h and APC infusion significantly reduced fluid accumulation in wildtype animals whereas it lacked a significant protective effect in PAR1 deficient mice (Fig. 6B). Histological analysis of the lungs performed on a subset of the animals revealed interstitial and focal alveolar edema in saline infused wild type mice as well as in both APC treated and untreated PAR1 deficient mice whereas APC infused wild type mice showed minimal to no alterations (Fig. 6C), consistent with the measurements of the wet to dry weight ratio. Interleukin-6 (IL-6) plasma levels were similarly high in all groups (Fig. 6D). These results indicate that APC leads to reduced fluid extravasation without affecting a marker of systemic inflammation showing that PAR1 plays a central role in APC mediated enhancement of endothelial barrier integrity in a sepsis model.

DISCUSSION

The mouse has become a unique and important tool for studying human diseases and for screening and testing putative drugs. However, species differences limit projecting conclusions drawn in one species onto the other. For example, thrombin signaling is mediated by PAR1 in human platelets and by PAR3 and PAR4 in mouse platelets (8). Here we demonstrate for the first time directly that mouse APC cleaves mouse PAR1 in endothelial cells. Using an antibody that blocks PAR1 cleavage-mediated signaling we provide evidence that APC can directly enhance the barrier of cultured endothelial cells by signaling through PAR1. We further show that APC-cleaved mouse PAR1 in contrast to thrombin cleaved mouse PAR1 is cell surface retained as described in human cell lines (20). Thus, PAR1 activation is linked to the protein C pathway similarly in humans and mice.

Previous studies in mouse models of cerebral ischemia demonstrated protective signaling of APC through PAR1 (10,27). Furthermore, APC treatment improved survival in an endotoxemia model in wildtype mice but not in PAR1-deficient mice (6). In wildtype mice APC treatment altered endotoxin-induced vascular hyperpermeability in the kidney, resulting in decreased permeability in small vessels but increased or unchanged permeability in medium sized or bigger vessels (6). However, this study did not analyze whether APC's effects on vascular permeability require PAR1. Here we extend these observations by demonstrating that in mouse models APC infusion protects from endotoxin-mediated fluid accumulation in a whole organ, i.e. the lung or the skin. In addition, we show for the first time that PAR1 plays a key role mediating the vascular barrier protective properties of infused APC *in vivo*. Taken together with our finding that PAR1-dependent APC signaling reduces monolayer permeability

in cultured mouse endothelial cells, these results strongly support the concept that PAR1 plays a major role mediating protective effects of APC on the vascular barrier integrity *in vivo*. How APC-PAR1 signaling leads to reduced permeability of the vascular barrier *in vivo* remains to be established. In tissue culture the endothelial barrier enhancing effect of APC requires the activity of cellular sphingosine kinase, sphingosine 1-phosphate (S1P)-receptor-1 transactivation, and activity of the small GTPase Rac1 (14,15). Given that S1P reduces the increased vascular permeability in animal models of acute inflammatory lung injury (28), it seems likely that S1P pathway- and Rac1-dependent changes of the organization of the endothelial actin cytoskeleton and cell-cell contacts also play a role in APC's vascular barrier protective activity *in vivo*.

Our results demonstrate that PAR1 plays a major role in mediating protective effects of infused APC on pulmonary fluid accumulation in a model of endotoxemia. The inflammatory response in this model of systemic inflammation includes strong upregulation of tissue factor expression on monocytic cells leading to thrombin generation (29). The reported PAR1 dependent effects could therefore either be direct by APC-PAR1 signaling or indirect by downregulation of thrombin generation and reduced thrombin-PAR1 signaling. To avoid such strong systemic responses we developed an animal model quantifying the dermal vascular barrier function. The dermis was chosen because of its easy accessibility, the high expression levels of EPCR and PAR1 within its vascular bed and its clinical relevance in protein C deficiency. VEGF acts directly on endothelial cells and mediates a well defined hyperpermeability response *in vitro* and *in vivo* (30). The finding that APC infusion protects vascular barrier integrity in both the endotoxemia and the skin model in a PAR1 dependent manner while PAR1-deficiency itself does not affect barrier integrity in either model, strongly argues that PAR1 signaling by the infused APC is beneficial. It remains to be established whether any remaining protective effects in the absence of PAR1 require APC's anticoagulant activity or are mediated by novel non PAR1-dependent APC signaling mechanisms in endothelial cells or other cell types such as leukocytes.

Given PAR1's role mediating vascular barrier protection by exogenously administered APC, it seems likely that endogenously generated APC also mediates protective effects through PAR1, especially in view of the finding that protein C activation on the endothelial surface is coupled to highly efficient PAR1-dependent barrier protective signaling by the generated APC in cultured endothelial cells (19). Our new data add to accumulating evidence that similar to thrombin itself the prototypical thrombin receptor PAR1 has dual-faced physiological roles, mediating both pro-inflammatory and protective effects. Originally PAR1 has been described as a receptor that mediates pro-inflammatory responses (31). Even though thrombin-PAR1 signaling is indeed pro-inflammatory in mouse models of glomerulonephritis (32), renal ischemia-reperfusion injury (33), and inflammatory bowel disease (34) PAR1 deficiency does not affect survival in mouse models of endotoxemia (6,35,36). Thus any beneficial effects of the absence of pro-inflammatory PAR1 signaling may be offset by the absence of cytoprotective PAR1-dependent signaling in the endotoxemia models. In a mouse sepsis model a pepducin PAR1 antagonist improved survival when the antagonist was administered immediately after surgery but not when given at later time points, whereas a PAR1 agonist was beneficial when given at late time points (37). These data indeed support the conclusion that PAR1 has opposite roles in systemic inflammation that may depend on the stage of the inflammatory response.

In conclusion, our results in complementary cell and mouse models demonstrate that PAR1 plays similar roles in endothelial barrier protective signaling by APC in humans and mice and they support the concept that APC by signaling through PAR1 maintains the integrity of the vascular barrier in systemic inflammation. The protein C pathway may have a role not only in

containing the thrombotic response but also in preventing excessive spreading of vascular barrier dysfunction during inflammation.

Acknowledgements

Grant support This study was supported by National Institutes of Health grants HL 73318 (to M.R.) and HL 31950 (to J.H.G.), grants from the University of Zurich, Switzerland (Zangger-Stiftung), the Swiss Foundation for Medical-Biological Grants (SSMBS) and the Mach-Gaensslen Stiftung (to R.A.S.), and an Austrian fellowship, Erwin Schrödinger-Auslandsstipendium J2413-B13 (to C.F.).

REFERENCES

1. Bernard GR, Vincent JL, Laterre PF, et al. Efficacy and safety of recombinant human activated protein C for severe sepsis. N Engl J Med 2001;344:699–709. [PubMed: 11236773]
2. Esmon CT. The protein C pathway. Chest 2003;124:26S–32S. [PubMed: 12970121]
3. Dahlback B, Villoutreix BO. Regulation of blood coagulation by the protein C anticoagulant pathway: novel insights into structure-function relationships and molecular recognition. Arterioscler Thromb Vasc Biol 2005;25:1311–1320. [PubMed: 15860736]
4. Warren BL, Eid A, Singer P, et al. Caring for the critically ill patient. High-dose antithrombin III in severe sepsis: a randomized controlled trial. Jama 2001;286:1869–1878. [PubMed: 11597289]
5. Abraham E, Reinhart K, Opal S, et al. Efficacy and safety of tifacogin (recombinant tissue factor pathway inhibitor) in severe sepsis: a randomized controlled trial. Jama 2003;290:238–247. [PubMed: 12851279]
6. Kerschen EJ, Fernandez JA, Cooley BC, et al. Endotoxemia and sepsis mortality reduction by non-anticoagulant activated protein C. The Journal of experimental medicine 2007;204:2439–2448. [PubMed: 17893198]
7. Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. Blood 2007;109:3161–3172. [PubMed: 17110453]
8. Coughlin SR. Thrombin signalling and protease-activated receptors. Nature 2000;407:258–264. [PubMed: 11001069]
9. Riewald M, Petrovan RJ, Donner A, et al. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. Science 2002;296:1880–1882. [PubMed: 12052963]
10. Guo H, Liu D, Gelbard H, et al. Activated protein C prevents neuronal apoptosis via protease activated receptors 1 and 3. Neuron 2004;41:563–572. [PubMed: 14980205]
11. Bae JS, Yang L, Rezaie AR. Lipid raft localization regulates the cleavage specificity of protease activated receptor 1 in endothelial cells. J Thromb Haemost 2008;6:954–961. [PubMed: 18284602]
12. Robriquet L, Collet F, Tournoys A, et al. Intravenous administration of activated protein C in Pseudomonas-induced lung injury: impact on lung fluid balance and the inflammatory response. Respir Res 2006;7:41. [PubMed: 16553944]
13. Abraham E. Effects of recombinant human activated protein C in human models of endotoxin administration. Proc Am Thorac Soc 2005;2:243–247. [PubMed: 16222045]
14. Feistritzer C, Riewald M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. Blood 2005;105:3178–3184. [PubMed: 15626732]
15. Finigan JH, Dudek SM, Singleton PA, et al. Activated protein C mediates novel lung endothelial barrier enhancement: role of sphingosine 1-phosphate receptor transactivation. J Biol Chem 2005;280:17286–17293. [PubMed: 15710622]
16. McVerry BJ, Garcia JG. Endothelial cell barrier regulation by sphingosine 1-phosphate. J Cell Biochem 2004;92:1075–1085. [PubMed: 15258893]
17. Peng X, Hassoun PM, Sammani S, et al. Protective effects of sphingosine 1-phosphate in murine endotoxin-induced inflammatory lung injury. American journal of respiratory and critical care medicine 2004;169:1245–1251. [PubMed: 15020292]

18. Feistritzer C, Lenta R, Riewald M. Protease-activated receptors-1 and -2 can mediate endothelial barrier protection: role in factor Xa signaling. J Thromb Haemost 2005;3:2798–2805. [PubMed: 16359518]
19. Feistritzer C, Schuepbach RA, Mosnier LO, et al. Protective signaling by activated protein C is mechanistically linked to protein C activation on endothelial cells. J Biol Chem 2006;281:20077–20084. [PubMed: 16709569]
20. Schuepbach RA, Feistritzer C, Brass LF, et al. Activated protein C-cleaved protease activated receptor-1 is retained on the endothelial cell surface even in the presence of thrombin. Blood 2007;111:2667–2673. [PubMed: 18089851]
21. Fernandez JA, Xu X, Liu D, et al. Recombinant murine-activated protein C is neuroprotective in a murine ischemic stroke model. Blood Cells Mol Dis 2003;30:271–276. [PubMed: 12737945]
22. Li W, Zheng X, Gu J, et al. Overexpressing endothelial cell protein C receptor alters the hemostatic balance and protects mice from endotoxin. J Thromb Haemost 2005;3:1351–1359. [PubMed: 15978090]
23. Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci U S A 1983;80:3734–3737. [PubMed: 6407019]
24. Riewald M, Ruf W. Protease-activated receptor-1 signaling by activated protein C in cytokine-perturbed endothelial cells is distinct from thrombin signaling. J Biol Chem 2005;280:19808–19814. [PubMed: 15769747]
25. Cantwell AM, Di Cera E. Rational design of a potent anticoagulant thrombin. J Biol Chem 2000;275:39827–39830. [PubMed: 11060281]
26. Gruber A, Fernandez JA, Bush L, et al. Limited generation of activated protein C during infusion of the protein C activator thrombin analog W215A/E217A in primates. J Thromb Haemost 2006;4:392–397. [PubMed: 16420571]
27. Cheng T, Liu D, Griffin JH, et al. Activated protein C blocks p53-mediated apoptosis in ischemic human brain endothelium and is neuroprotective. Nat Med 2003;9:338–342. [PubMed: 12563316]
28. McVerry BJ, Garcia JG. In vitro and in vivo modulation of vascular barrier integrity by sphingosine 1-phosphate: mechanistic insights. Cell Signal 2005;17:131–139. [PubMed: 15494205]
29. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001;13:85–94. [PubMed: 11257452]
30. Weis SM, Cheresh DA. Pathophysiological consequences of VEGF-induced vascular permeability. Nature 2005;437:497–504. [PubMed: 16177780]
31. Coughlin SR, Camerer E. PARticipation in inflammation. J Clin Invest 2003;111:25–27. [PubMed: 12511583]
32. Cunningham MA, Rondeau E, Chen X, et al. Protease-activated receptor 1 mediates thrombin-dependent, cell-mediated renal inflammation in crescentic glomerulonephritis. The Journal of experimental medicine 2000;191:455–462. [PubMed: 10662791]
33. Sevastos J, Kennedy SE, Davis DR, et al. Tissue factor deficiency and PAR-1 deficiency are protective against renal ischemia reperfusion injury. Blood 2007;109:577–583. [PubMed: 16990608]
34. Vergnolle N, Cellars L, Mencarelli A, et al. A role for proteinase-activated receptor-1 in inflammatory bowel diseases. J Clin Invest 2004;114:1444–1456. [PubMed: 15545995]
35. Pawlinski R, Pedersen B, Schabbauer G, et al. Role of tissue factor and protease-activated receptors in a mouse model of endotoxemia. Blood 2004;103:1342–1347. [PubMed: 14576054]
36. Camerer E, Cornelissen I, Kataoka H, et al. Roles of protease-activated receptors in a mouse model of endotoxemia. Blood 2006;107:3912–3921. [PubMed: 16434493]
37. Kaneider NC, Leger AJ, Agarwal A, et al. 'Role reversal' for the receptor PAR1 in sepsis-induced vascular damage. Nat Immunol 2007;8:1303–1312. [PubMed: 17965715]

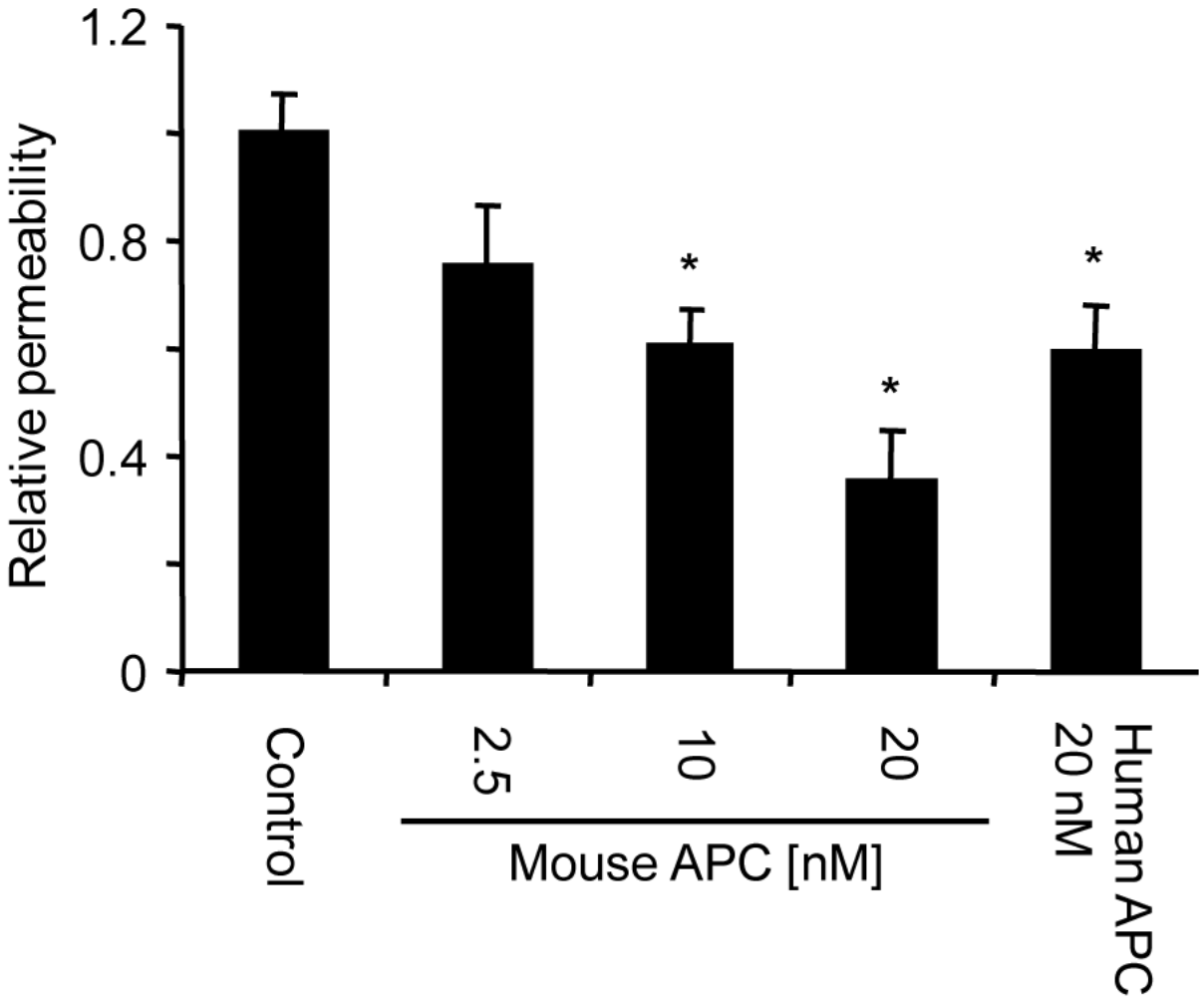

Figure 1. Effect of APC on endothelial barrier permeability in mouse endothelial cells in tissue culture

Subconfluent murine endothelial cells (b.End3) were incubated for 3 h with the indicated agonists in a dual chamber system followed by analysis of permeability. Values are means ±SEM, n=6 to 10, *P<0.05.

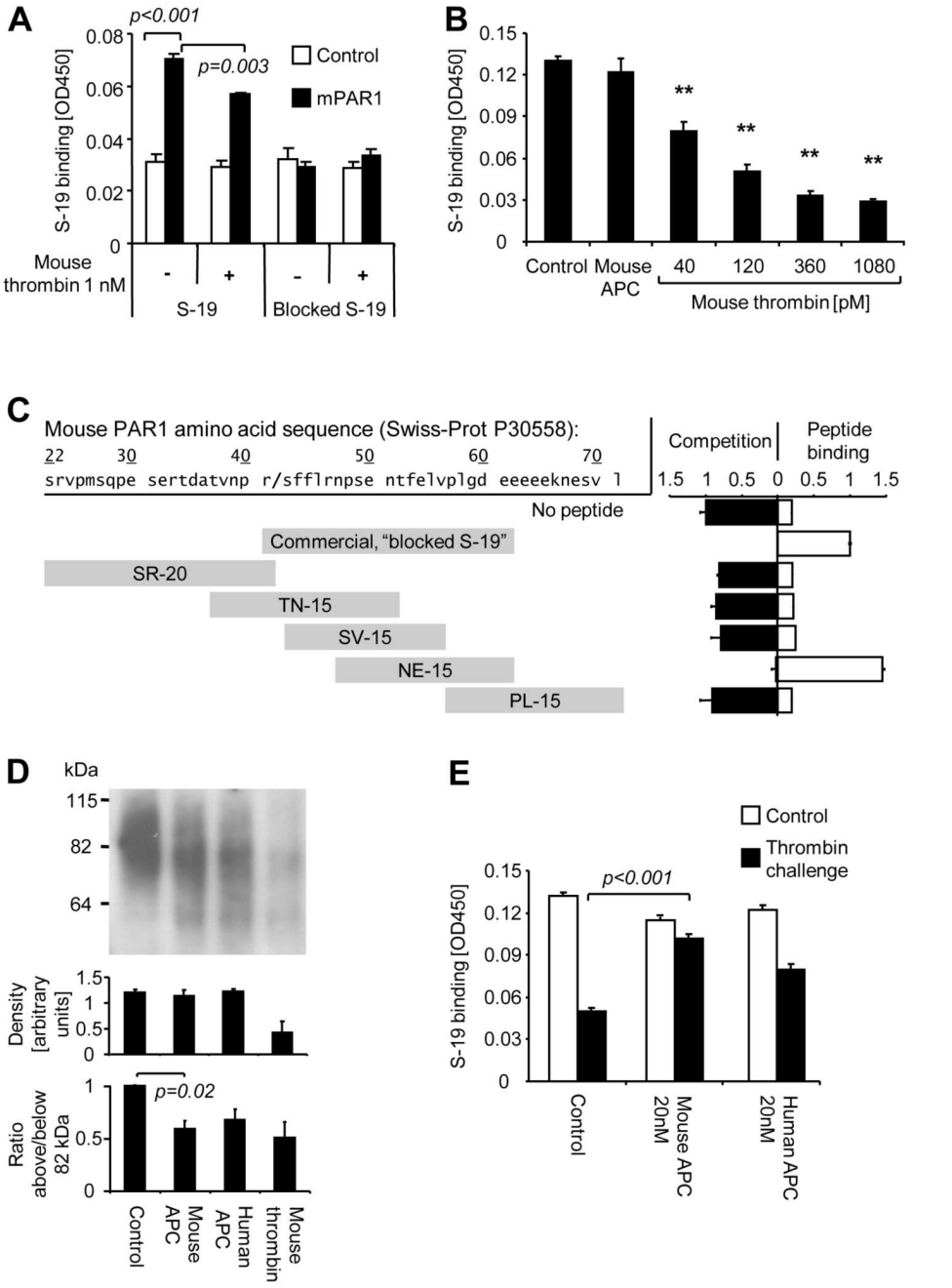

Figure 2. Cleavage of cell surface-exposed mouse PAR1 by APC

A) The human EA.hy926 endothelial cells were transfected with cDNA encoding mouse PAR1 (mPAR1) or control cDNA followed two days later by a 30 minutes agonist incubation. Cell surface binding of S-19 anti-mouse PAR1 and immunizing peptide-blocked S-19 is shown. B) Expression of PAR1 on the surface of a monolayer of murine MS1 endothelial cells was analyzed after a 3 h incubation with the indicated agonists by cell surface ELISA using anti-mouse PAR1 S-19. APC was used at 20 nM. C) The indicated peptides corresponding to the N-terminal amino acid sequence of mouse PAR1 were analyzed for binding to S-19. In a competition assay S-19 binding to cell surface expressed PAR1 was tested in the presence of excess peptide. Direct binding of S-19 to immobilized peptides was analyzed in a peptide

binding assay. D) Following a 3 h incubation with vehicle, 20nM of APC or 1nM of mouse thrombin the cell surface proteins were biotinylated and PAR1 was immunoprecipitated using S-19. Biotinylated proteins were detected on blots with peroxidase-coupled streptavidin. Bands are shown from a representative experiment in the upper panel and density between 55 and 120 kDa was quantified in three independent repeat experiments (middle panel). The ratio of density above and below PAR1's native average size of 82 kDa is shown in the lower panel. E) MS1 cells were incubated for 3 h with control or APC followed by an additional 30 min incubation with vehicle alone or mouse thrombin (0.5 nM). Binding of S-19 anti-PAR1 was analyzed by cell surface ELISA. Means±SEM are shown in all panels, n=3 (A), 4 (B), 3 (C,D) and 16 (E), *P* values are indicated.

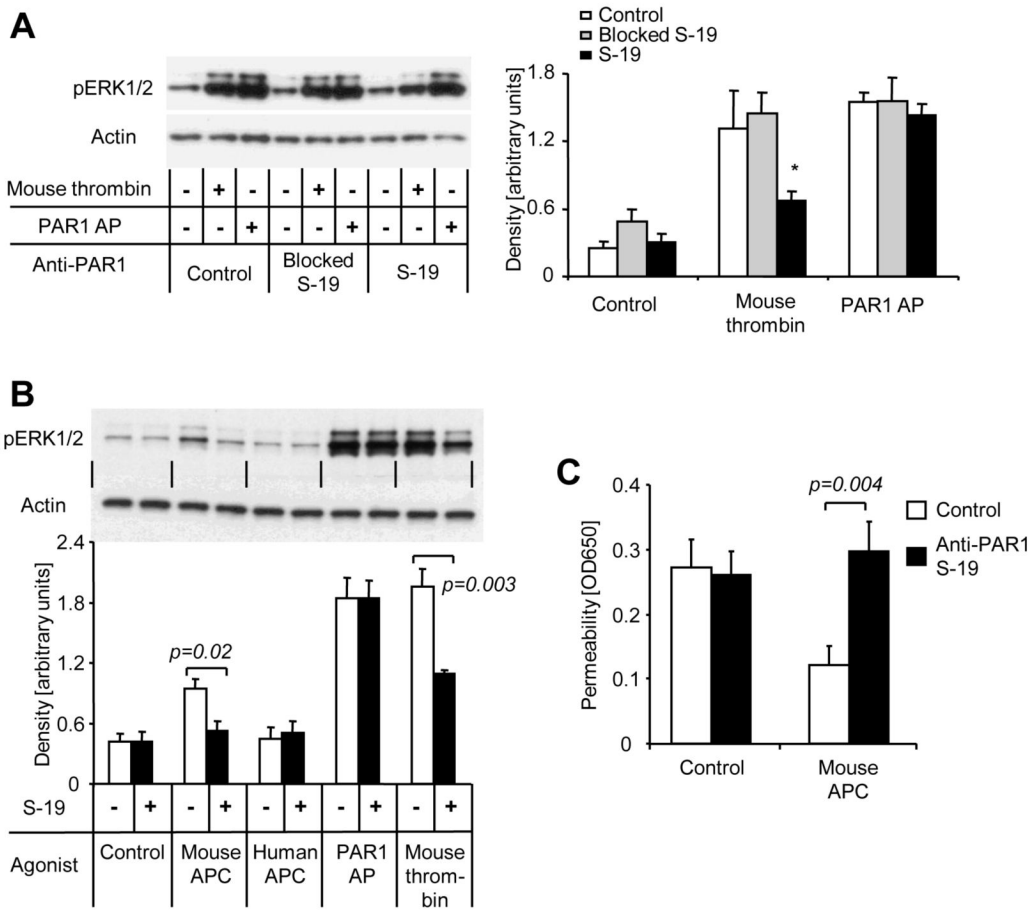

Figure 3. Anti-PAR1 S-19 blocks PAR1 signaling and APC-mediated barrier protection in mouse endothelial cells

A) ERK1/2 phosphorylation was analyzed after 7 min incubation with mouse thrombin (1 nM) or the PAR1 agonist peptide TFLLRNPNDK (PAR1 AP; 20 μM). Where indicated anti PAR1 S-19 (25 μg/ml) alone or blocked by preincubation with excess of immunizing peptide was added 15 min prior to the agonists. A representative blot is shown on the left side, quantitative analyzes of S-19 immunoreactive bands are given in the right part of the figure (means±SEM) and ANOVA reviled that S-19 only significantly affects the thrombin response (**P*<0.05). B) ERK1/2 phosphorylation in response to the indicated agonists (7 min) was analyzed in the absence or presence of S-19. APC was used at 20 nM. Representative blot shown in the upper part, quantification (means±SEM) of 4 independent experiments in the lower part, *P* value indicated. C) Subconfluent murine endothelial cells (b.End3) were incubated for 3 h with mouse APC (20 nM) in the absence or presence of S-19 in a dual chamber system followed by analysis of permeability. Means±SEM, n=9; *P* value indicated.

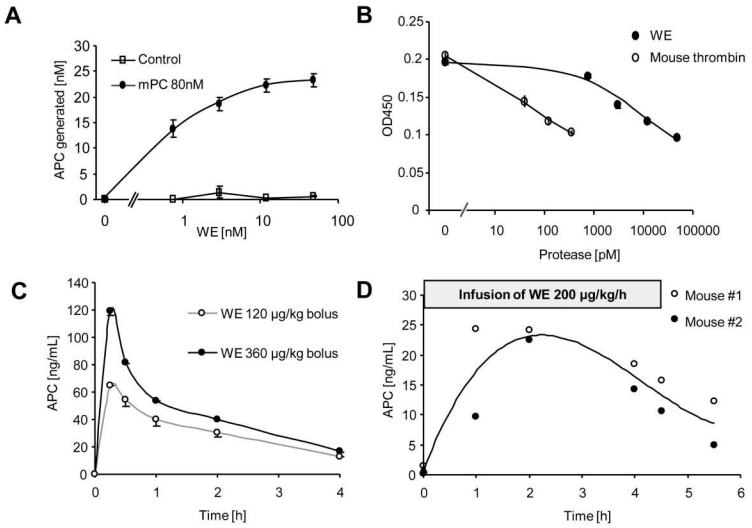

Figure 4. Human anticoagulant mutant thrombin (WE) efficiently activates mouse protein C in *vitro* and *in vivo* but poorly activates PAR1

A) Mouse endothelial cells (MS1) were incubated for 3 h with indicated concentrations of WE in the presence and absence of 80 nM of mouse protein C (means±SEM, n=6). B) Expression of PAR1 on the surface of a monolayer of murine MS1 endothelial cells was analyzed after a 3 h incubation with the indicated agonists by cell surface ELISA using anti-mouse PAR1 S-19 (means±SEM, n=3). C) Plasma APC levels after a bolus injection of the indicated amounts of WE (means±SEM, n=3) D) In two mice plasma APC levels were assessed immediately before, during and after a 4.5 h infusion with WE.

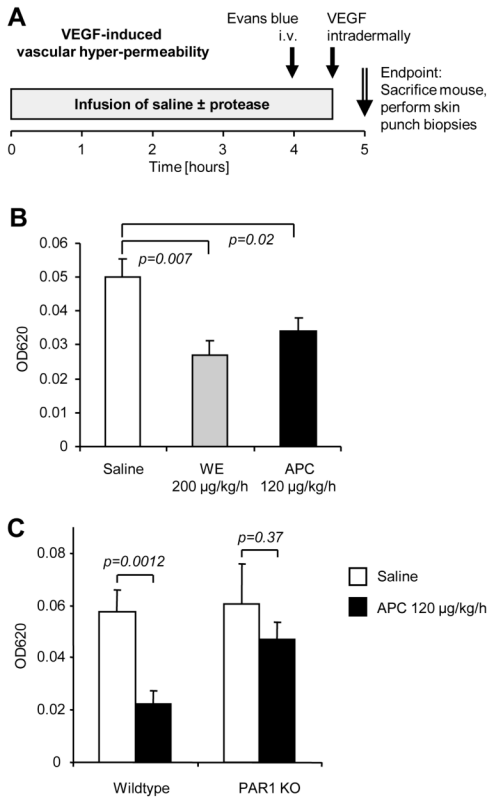

Figure 5. PAR1 dependent vascular barrier protection by APC in a modified Miles assay

A) Male mice were infused with saline, murine APC or WE for 4.5 h and Evans blue was intravenously injected 30 min prior to the end of the infusion. At the end of the infusion 25 ng of murine VEGF was intradermally injected and 30 min later the injection sites were removed by punch biopsies. B) C57BL/6 wildtype mice were infused in parallel. Evans blue content in VEGF-injected skin is shown. C) Evans blue content of an additional set of parallel infused C57BL/6 wildtype and PAR1 deficient (PAR1 KO) animals. Means±SEM are shown in all panels, n=10 per group (B, C), *P* values are indicated.

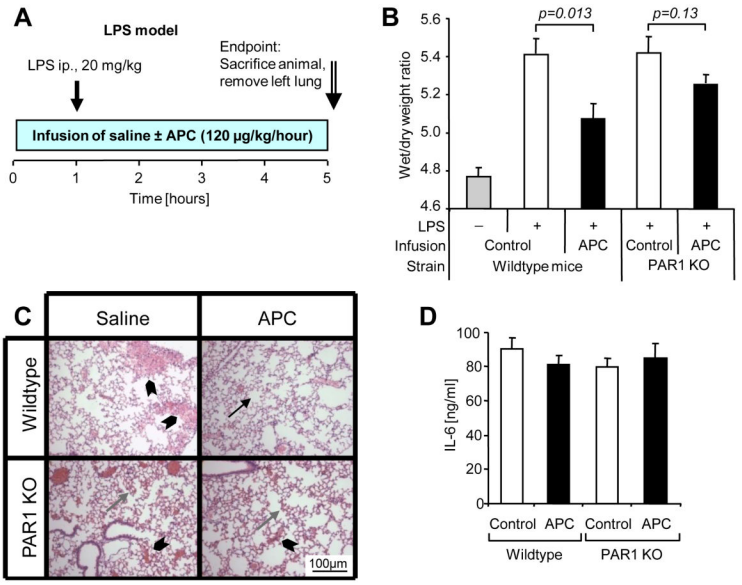

Figure 6. Infusion of APC reduces pulmonary fluid accumulation in a mouse endotoxemia model dependent on PAR1

A) Male C57BL/6 wildtype or PAR1-deficient (PAR1 KO) mice were infused with either saline alone or with murine APC (120 µg/kg/h) for 5 h via a central venous catheter (10-12 mice per group). LPS (20 mg/kg) was injected intraperitoneally one hour after the start of the infusion. Lungs were dissected for wet/dry weight ratio determination at the end of the infusion period. B) Lung wet weight/dry weight ratios are shown (means±SEM, *P* values indicated). Results from non LPS injected animals are shown as a reference. C) Hematoxylin-Eosin stained representative pulmonary tissue sections show normal alveolar tissue (arrow) alveolar edema (gray arrow) and alveolar flooding (arrowhead). D) IL-6 plasma levels at the end of the infusion are shown (means±SEM).

Journal of Thrombosis and Haemostasis, **8**: 379–388 DOI: 10.1111/j.1538-7836.2009.03682.x

ORIGINAL ARTICLE

Coagulation factor Xa cleaves protease-activated receptor-1 and mediates signaling dependent on binding to the endothelial protein C receptor

R. A. SCHUEPBACH and M. RIEWALD
Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA, USA

To cite this article: Schuepbach RA, Riewald M. Coagulation factor Xa cleaves protease-activated receptor-1 and mediates signaling dependent on binding to the endothelial protein C receptor. *J Thromb Haemost* 2010; **8**: 379–88.

Summary. *Background and objective:* Coagulation is intrinsically tied to inflammation, and both proinflammatory and anti-inflammatory responses are modulated by coagulation protease signaling through protease-activated receptor-1 (PAR1). Activated factor X (FXa) can elicit cellular signaling through PAR1, but little is known about the role of cofactors in this pathway. Endothelial protein C receptor (EPCR) supports PAR1 signaling by the protein C pathway, and in the present study we tested whether EPCR mediates surface recruitment and signaling of FXa. *Methods and results:* Here, we show that FXa binds to overexpressed as well as native endothelial EPCR. PAR1 cleavage by FXa as analyzed with conformation-sensitive antibodies and a tagged PAR1 reporter construct was strongly enhanced if EPCR was available. Anti-EPCR failed to affect the tissue factor-dependent activation of FX, but high concentrations of FXa decreased EPCR-dependent protein C activation. Most importantly, the FXa-mediated induction of Erk1/2 activation, expression of the transcript for connective tissue growth factor and barrier protection in endothelial cells required binding to EPCR. *Conclusions:* Our results demonstrate that EPCR plays an unexpected role in supporting cell surface recruitment, PAR1 activation, and signaling by FXa.

Keywords: endothelial cells, endothelial protein C receptor, factor Xa, protease-activated receptor-1.

Introduction

Factor X, the zymogen clotting factor, is catalytically activated to the active serine protease (FXa) upon binding to the cell surface complex formed by tissue factor and protease ligand activated FVII (FVIIa) [1]. If the ternary complex is not inhibited by tissue factor pathway inhibitor [2], FXa will dissociate, assemble with cofactor activated clotting factor V (FVa) on cell surfaces, and proteolytically activate prothrombin to thrombin, which ultimately will allow clot formation by cleaving fibrinogen and activating platelets. Thrombin in complex with the cell surface receptor thrombomodulin also activates the anticoagulant protein C pathway in a negative feedback loop [3].

Beyond directly controlling coagulation, clotting factors have non-hemostatic signaling functions that play important roles in physiology and disease. Coagulation factors induce signal transduction through protease-activated receptors (PARs). These closely related G-protein-coupled receptors allow cells to sense for proteolytic activity in their microenvironment [4]. Whereas thrombin, a major mediator of cell-signaling events, directly binds to, cleaves and activates its prototypical receptor PAR1, other clotting factors require coreceptor binding. FVIIa has been shown to induce PAR2-mediated signaling dependent on binding to tissue factor [5]. Activated protein C (APC) proteolytically activates PAR1 and induces cytoprotective signaling in endothelial cells dependent on binding to endothelial protein C receptor (EPCR) [6,7]. EPCR is a cell membrane glycoprotein that shares homology with the CD1 family of major histocompatibility complex class 1 molecules. Binding of protein C and APC to EPCR is Ca^{2+}-dependent and involves the γ-carboxyglutamic acid (Gla) domain of protein C [8,9].

Cellular signaling by FXa has been implicated in a variety of conditions, including wound healing and tissue fibrosis, atherosclerosis and restenosis, airway remodeling, cancer dissemination, and angiogenesis (see [10] for a review). In view of these findings, therapeutic targeting of cellular signaling by FXa seems to be a promising concept, even though many *in vitro* studies have used relatively high FXa concentrations, and a precise pathological and/or physiological role of FXa signaling *in vivo* remains to be established. Accordingly, a large number of studies have addressed the question of how cellular FXa signaling is mediated. Previous studies have shown that FXa can activate both PAR1 and PAR2 on a variety of different cell types, including endothelial cells [10]. FXa in a ternary complex with tissue factor and FVIIa can more

Correspondence: Matthias Riewald, The Scripps Research Institute, Department of Immunology and Microbial Science, SP30-3040, 10550 North Torrey Pines Rd, La Jolla, CA 92037, USA.
Tel.: +1 858 784 8226; fax: +1 858 784 7545.
E-mail: riewald@scripps.edu

Received 12 May 2009, accepted 3 November 2009

efficiently signal through both PAR1 and PAR2 [11], but other cofactors have also been implicated [12].

Recent studies have shown that the Gla domains of not only APC but also of FVIIa can bind to EPCR [13–15]. Given that FXa also contains a highly homologous Gla domain, we hypothesized that FXa, APC and FVIIa might share one or more coreceptors for endothelial cell surface binding, and that EPCR might be one of them. Here, we show that FXa does indeed bind to endogenously expressed EPCR, and that cleavage of PAR1 by FXa is strongly enhanced in the presence of EPCR. Consistent with EPCR-dependent activation of PAR1, induction of extracellular signal-related kinase (ERK)1/2 phosphorylation and expression of connective tissue growth factor (CTGF) in an endothelial cell line were also found to depend on EPCR.

Materials and methods

Reagents

Human thrombin and PAR1 and PAR2 agonist peptides were as previously described [6,11]. Clotting proteases blocked with phenylanalyl-prolyl-arginyl-chloromethylketone (FPRCK; for thrombin), dansyl-glutamyl-glycyl-arginyl-chloromethylketone (DEGR; for APC) and biotinylated-glutamyl-glycyl-arginyl-chloromethylketone (bEGR; for Xa and APC) were obtained from Haematologic Technologies (Essex Junction, VT, USA), with the exception of Gla domainless APC (Enzyme Research Laboratories, South Bend, IN, USA). All experiments involving stimulation with FXa, APC or FVIIa included hirudin (Calbiochem, La Jolla, CA, USA), unless indicated otherwise. Control experiments demonstrated that hirudin alone had no effect in any of our assays. Monoclonal anti-PAR1 ATAP2 was as previously described [16], and SPAN12/5 was recloned from SPAN12 hybridoma cells that were kindly provided by L. Brass [17]. Monoclonal rat anti-EPCR RCR92 (non-blocking) and RCR252 (blocking Gla-domain binding to EPCR) were kindly provided by K. Fukudome (Saga Medical School, Saga, Japan) and were used at 25 μg mL^{-1} [18]. Amidolytic assays for APC and FXa activity were as described previously [19]. The recombinant FXa inhibitor nematode anticoagulant protein 5 (NAP5) was provided by G. Vlasuk [20].

Cell culture and transfection

EA.hy926 cells [21] and primary human umbilical vein endothelial cells (HUVECs; Cascade Biologics, Portland, OR, USA) were cultivated as described previously [16,22]. In experiments involving gene silencing, cells were plated together with complexes of small interfering RNA (siRNA; 30 pM final concentration) and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Cells were used for experiments 48 h after transfection, and the tissue culture medium was replaced on the day before the experiment. Chemically synthesized, double-stranded siRNA with 19-nucleotide duplex RNA and two-nucleotide 3′-dTdT overhangs was obtained from Ambion (Austin, TX, USA). The siRNA sequences were GGGAAUAUUGCCAAUGCUAtt (targeting PAR1), CAACCGCACUCGGUAUGAAtt (targeting EPCR), and GGAUCAAACUCUGCUUCCUtt (targeting PAR4 and used as a control). Real-time polymerase chain reaction analysis of PAR1, PAR2 and EPCR mRNA levels was used to demonstrate the efficiency of downregulation of the specific target and to rule out non-specific effects on other genes. CHO-K1 and HEK293 cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (for CHO-K1 cells; Invitrogen) or DMEM (for HEK293 cells; Invitrogen). Both media were supplemented with 10% fetal bovine serum. The cells were transiently transfected with the pcDNA3.1/Zeo+ plasmid vector (Invitrogen), using Lipofectamine 2000. The expression constructs containing the human PAR1 or EPCR coding sequences were as described previously [6,11]. To obtain a PAR1 cleavage reporter construct, the coding sequence of PAR1 was cloned (*Eco*RI/*Xho*I restriction sites) into a modified pcDNA3.1/Zeo+ vector with a deleted *Apa*I site. The signal peptide of PAR1 was removed using *Eco*RI and PAR1's native *Apa*I site, and replaced by secretory alkaline phosphatase (SEAP). The SEAP coding sequence containing a 5′ *Eco*RI site and a frame-adjusted 3′ *Apa*I site was a kind gift from L. Mosnier [23]. HEK293 cells were used for experiments 24 h after transfection. To obtain CHO-K1 cells stably expressing EPCR, the pcDNA3.1/Hygro+ plasmid containing the coding sequence for EPCR was linearized with *Sap*I and transfected into CHO-K1 cells. Corresponding empty vector was used to generate control cells. Two days after transfection, selection with hygromycin (800 μg mL^{-1}) was initiated. Untransfected CHO-K1 cells were found to be eliminated within 5 days if exposed to 400 μg mL^{-1} hygromycin. After 10 days of selection, surviving cells were cloned, and a clone with normal growth speed and high and stable EPCR expression was used for experiments.

Determination of surface binding of proteases

Cell surface protease binding was quantified using a modified method described by Ghosh *et al.* [14]. Briefly, confluent cell layers were washed once with Ca^{2+}-free Hank's buffered salt solution (HBSS) containing 5 mM EDTA, and then with HBSS containing 1% bovine serum albumin (BSA), 1 mM $MgCl_2$ and 5 mM $CaCl_2$, and kept in this buffer for all subsequent steps. Cells were chilled and kept on ice throughout the experiment. Following incubation with biotinylated proteases for 3 h, the cells were washed twice, incubated for 10 min with horseradish peroxidase (HRP)-coupled streptavidin (Invitrogen), and washed again five times before tetramethylbenzidine was added for spectrophotometric quantification. Negatively charged phospholipid-rich cell membrane domains were quantified using biotinylated annexin V (Calbiochem) instead of biotinylated protease in the same assay with a 15-min incubation time.

Cell surface immunoassays, western blotting, and real-time PCR

Cell surface PAR1 and EPCR were quantified by cell surface enzyme-linked immunosorbent assay as described previously [16]. PAR1 was detected with biotinylated (Mini-Biotin-XX Protein Labeling Kit; Invitrogen) mouse monoclonal SPAN12/5 (2 μg mL^{-1}) or ATAP2. EPCR was detected with biotinylated rat monoclonal RCR252. Streptavidin-coupled HRP and tetramethylbenzidine were used for spectrophotometric quantification of cell surface antibody binding. Immunoblotting was performed as previously described [16]. Mitogen-activated protein (MAP) kinase phosphorylation was detected with rabbit anti-phospho-ERK1/2 (#9101; Cell Signaling Technology, Beverly, MA, USA), as described previously [24]. The optical density of immunoreactive bands was quantified using Scion Image Alpha 4.0.3.2 software (Scion Corporation, Frederick, MD, USA). Quantification of mRNA encoding EPCR or CTGF by real-time PCR was as previously described [25] and normalized to glyceraldehyde-3-phosphate dehydrogenase transcript levels.

Permeability assay

Macromolecular monolayer permeability was analyzed in a dual chamber system, using Evans blue-labeled BSA as described previously [22,26]. Briefly, EA.hy926 cells were plated on Transwell polycarbonate membranes of 3-μm pore size and 12-mm diameter (#3402; Corning Inc., Corning, NY, USA). The upper and lower chambers were filled with 500 μL and 1500 μL, respectively, of growth medium. Cells were grown for 2 days, agonists were added in serum-free medium containing 0.4% BSA, and permeability was assayed 3 h later, using 0.67 mg mL^{-1} Evans blue diluted in growth medium containing 4% BSA. Fresh growth medium was added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. After 10 min, the optical density at 650 nm was measured in a 1 : 3 diluted 50-μL sample from the lower chamber.

PAR1 cleavage reporter assay

HEK293 cells transiently expressing alkaline phosphatase (AP)-tagged PAR1 were washed twice and incubated with agonists for 20 min. The supernatants were removed, and separated from cell debris by passing them through a cellulose ester filter (pore size, 0.45 μm); AP activity was then quantified using the colorimetric substrate *p*-nitrophenyl phosphate (1-Step PNPP; Thermo Scientific, Rockford, IL, USA).

Statistical analysis

Data analysis was performed using NCSS Statistical and Power Analysis or SigmaStat 3.5 (Systat Software Inc., Chicago, IL, USA) software. A two-sample, two-tailed homoscedastic *t*-test was used to calculate the indicated *P*-values.

Results

Overexpressed EPCR supports FXa binding to the cell surface

In order to investigate whether EPCR can recruit FXa to the cell surface, we analyzed the binding of active site-blocked biotinylated human FXa and APC to CHO-K1 cells. Cells were incubated at 4 °C to prevent internalization of the bound proteases. Binding to native CHO-K1 cells and mock-transfected control cells was very low, indicating that CHO-K1 cells do not significantly express high-affinity receptors for the human proteases. Unexpectedly, CHO cells stably expressing human EPCR (CHO-EPCR cells) bound FXa and APC with comparable efficiency (Fig. 1A). Similar results were obtained in transiently transfected HEK293 cells (not shown). Confluent monolayers of CHO-EPCR cells had expression levels of EPCR approximately three times higher than that of native EPCR on human endothelial EA.hy926 cells (Fig. 1B). FXa and APC binding to CHO-EPCR cells was Ca^{2+}-dependent (Fig. 1C). The Gla domain-containing coagulation proteases can bind to negatively charged phospholipids in the cell membrane in a Ca^{2+}-dependent manner. We therefore used annexin V, which specifically binds to anionic phospholipids, to test whether CHO-EPCR cells express higher levels of these binding sites. As shown in Fig. 1D, annexin V binding was comparable in CHO-K1, mock-transfected and CHO-EPCR cells. Furthermore, active site-blocked biotinylated thrombin bound similarly to control and EPCR-expressing cells (Fig. 1E). Taken together, these data indicate that human EPCR can support the Ca^{2+}-dependent cell surface recruitment of FXa. Active site-blocked APC, zymogen protein C, zymogen FX and FVIIa all competed for FXa binding to CHO-EPCR cells, consistent with the conclusion that FXa shares binding sites with APC and FVIIa (Fig. 1F). In contrast, protein S, FVa and active site-blocked thrombin did not compete with FXa–EPCR binding to CHO-EPCR cells. The absence of competition by the Gla domain-containing protein S indicates that non-specific competition by the Gla domain does not explain the results obtained using protein C/APC, FX, and FVIIa.

Reduced FXa binding to the endothelial cell surface in the presence of APC or anti-EPCR

Next, we analyzed FXa binding to human endothelial cell lines. Binding of biotinylated FXa and APC to EA.hy926 cells was time-dependent (not shown) and dose-dependent (Fig. 2A), and half-maximal binding was reached for both proteases at concentrations of approximately 30 nM. FXa binding to primary HUVECs was comparable (Fig. 2B). Binding was again Ca^{2+}-dependent, because almost no surface binding was detected in the absence of Ca^{2+} in the binding buffer or in the presence of EDTA (data not shown). To determine whether the endothelial surface binding sites for FXa are shared with other clotting factors, we analyzed FXa binding in the presence of a 100-fold molar excess of unlabeled FX, active site-blocked

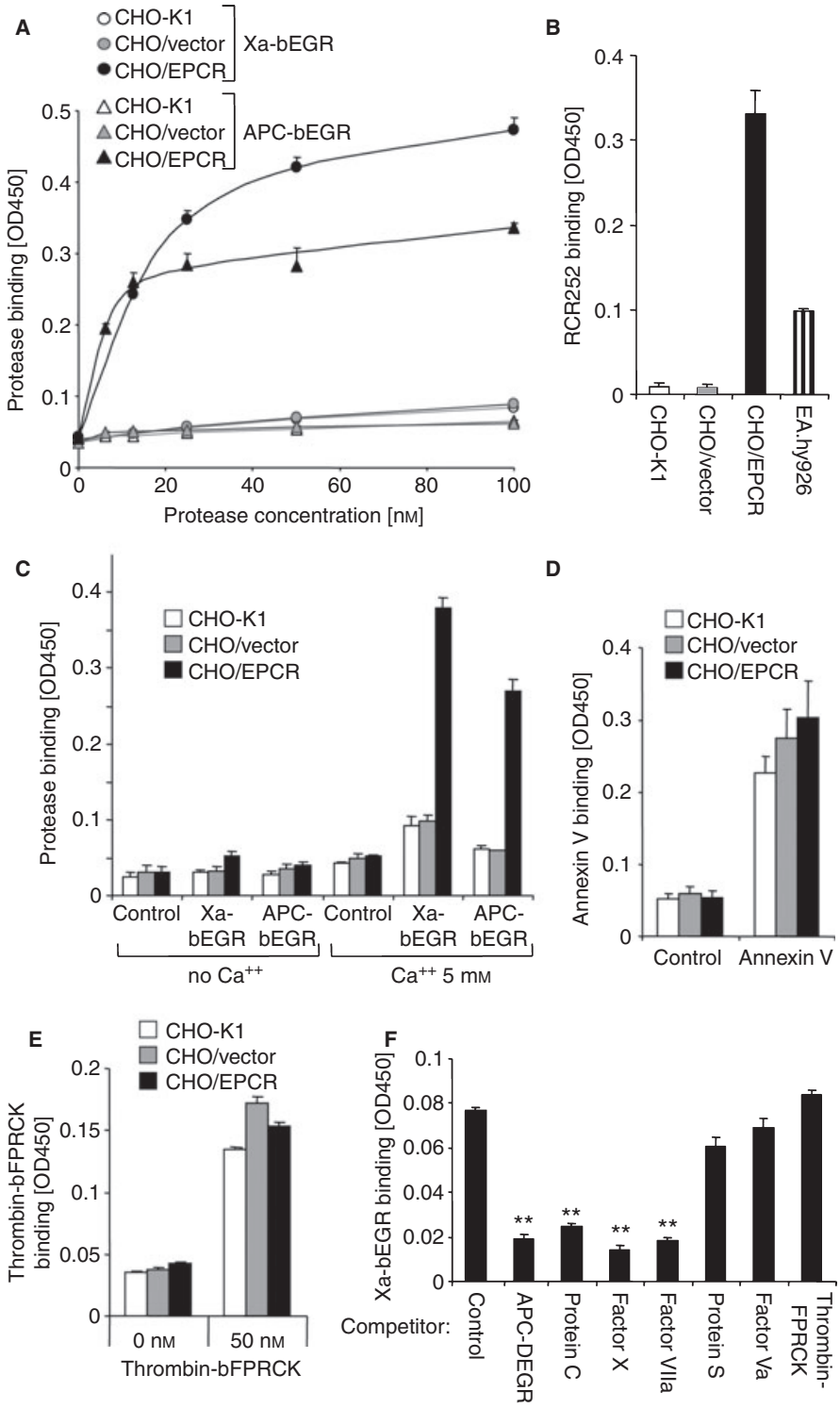

Fig. 1. Binding of biotinylated active site-blocked FXa to overexpressed endothelial protein C receptor (EPCR). (A) CHO-K1 cells either non-transfected or stably expressing empty vector or human EPCR were incubated for 3 h at 4 °C with the indicated concentrations of biotinylated active site-blocked FXa (FXa–bEGR) or activated protein C (APC) (APC–bEGR) and the amount of surface-associated proteases was determined. (B) Quantification of monoclonal rat anti-EPCR (RCR252) binding to the indicated cell lines. (C) Binding of 50 nM FXa–bEGR or APC–bEGR was quantified in the absence and presence of Ca^{2+}. (D) Binding of biotinylated annexin V to the indicated cell lines was analyzed. (E) Binding of biotinylated active site-blocked thrombin was quantified. (F) In CHO-K1 cells stably expressing EPCR, the surface binding of 10 nM FXa–bEGR was analyzed in the absence or presence of 1 μM unlabeled active site-blocked APC (APC–DEGR), active site-blocked thrombin (thrombin–FPRCK), and other coagulation factors as indicated. Results in all panels are expressed as means ± standard errors of the mean. (A–C, F) $n = 6$; (D) $n = 5$; (E) $n = 12$. $^{**}P < 0.005$ as compared with control.

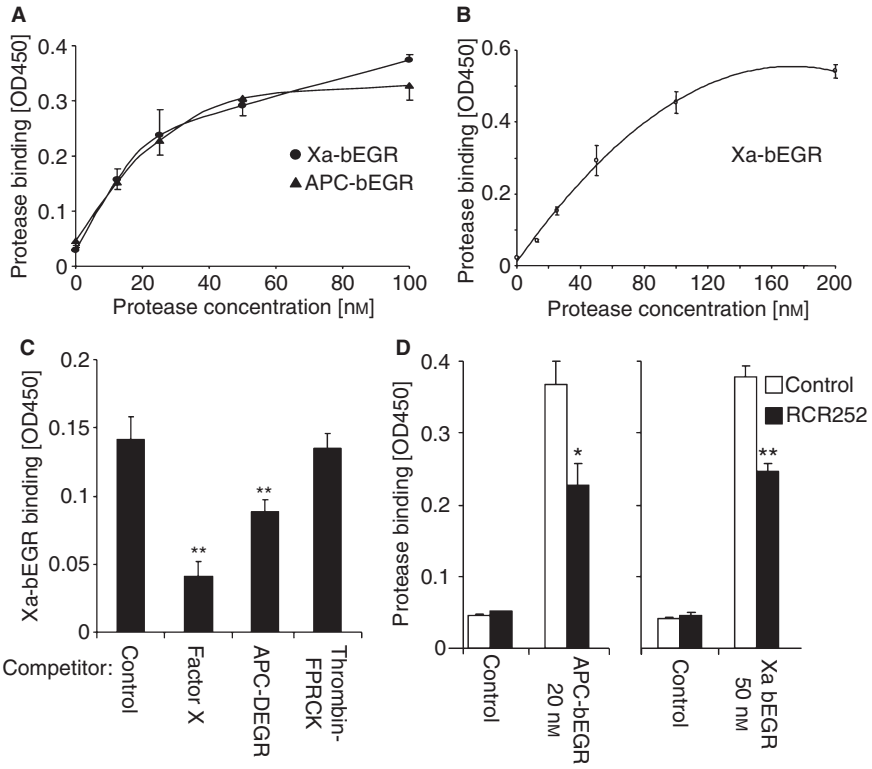

Fig. 2. Binding of FXa–bEGR to the endothelial cell surface. EA.hy926 cells (A) or human umbilical vein endothelial cells (B) were incubated for 3 h at 4 °C with the indicated concentrations of FXa–bEGR or activated protein C (APC)–bEGR, and this was followed by analysis of surface binding. (C) Surface binding of 10 nM FXa–bEGR to EA.hy926 cells was analyzed in the absence or presence of 1 μM of the indicated competitors. (D) Cells were preincubated in the absence or presence of 25 μg mL^{-1} the endothelial protein C receptor-blocking antibody RCR252 before the 3-h incubation at 4 °C in the absence or presence of APC–bEGR or FXa–bEGR. Results are expressed as means ± standard errors of the mean. (A, D) $n = 3$; (B) $n = 5$; (C) $n = 6$. $^*P < 0.05$ and $^{**}P < 0.005$ as compared with control. DEGR; FPRCK.

APC, or active site-blocked thrombin. FXa binding was significantly reduced in the presence of FX or APC, whereas thrombin did not compete for FXa binding (Fig. 2C). The finding that competition by FX was more efficient than that by APC may indicate that affinities for a shared receptor on endothelial cells are different or that one or more receptors that support FX/Xa binding do not bind APC. To test whether EPCR constitutes binding sites that are shared by FXa and APC, we used the RCR-252 monoclonal anti-EPCR, which is known to block the interaction of APC with EPCR. RCR-252 did indeed reduce surface binding of both APC and FXa to similar degrees (Fig. 2D), suggesting that EPCR is a major binding receptor for FXa on the EA.hy926 cell surface.

Gla-domain-dependent cleavage of endothelial PAR1 by FXa

Cell surface immunoassays were used to quantify cleavage of endogenous endothelial PAR1 by FXa. We have previously established that ATAP2 is conformation-sensitive and does not bind to cleaved PAR1 in the immunoassay [16]. SPAN12/5 anti-PAR1 was raised against a peptide spanning the cleavage site, and is expected to be cleavage-sensitive [17]. Small interfering RNA targeting PAR1 reduced PAR1 message and antigen to < 5% (data not shown). Cell surface binding of SPAN12/5 was reduced close to baseline if cells were pretreated with siRNA targeting PAR1 or if the SPAN12/5 epitope on PAR1's N-terminus was removed by the specific agonist thrombin, demonstrating that SPAN12/5 specifically binds to full-length uncleaved PAR1 (Fig. 3A). A 3-h incubation with both FXa and APC significantly reduced binding of monoclonal anti-PAR1 ATAP2 and SPAN12/5 (Fig. 3B). As expected, FVIIa had no effect on anti-PAR1 binding. The downregulation of SPAN12 staining after a 30-min incubation with FXa as well as APC was dose-dependent, with highly significant effects already being seen at a protease concentration of 12.5 nM. The Gla-domain-deficient corresponding proteases had amidolytic activity similar to the that of the wild type (data not shown), but did not downregulate SPAN12/5 staining at up to 50 nM (Fig. 3C). The FXa inhibitor NAP5 blocked FXa but not APC responses, demonstrating specificity and the dependence on FXa's proteolytic activity (Fig. 3D). These results directly demonstrate, for the first time, that FXa leads to cleavage of native endothelial PAR1 dependent on its Gla domain and proteolytic activity.

EPCR supports PAR1 cleavage by FXa

After having established an assay that allows the specific and sensitive quantification of PAR1 cleavage by FXa, and in view of our evidence for substantial binding of FXa to EPCR, we

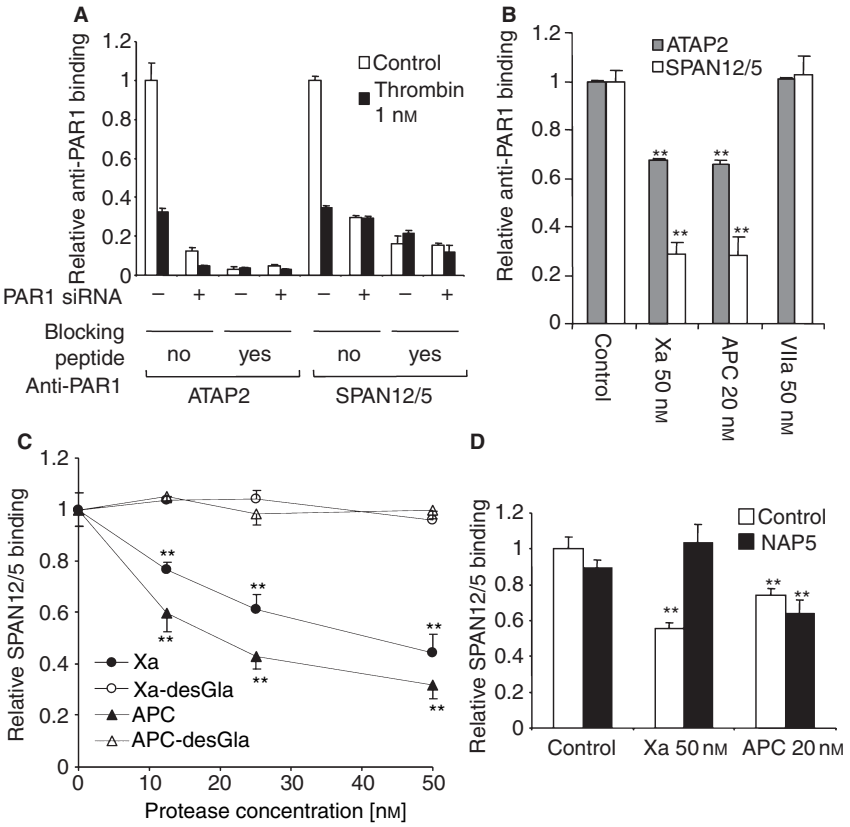

Fig. 3. Quantification of cleavage of endogenous endothelial protease-activated receptor-1 (PAR1) by FXa using a SPAN12/5-based immunoassay. (A) EA.hy926 endothelial cells were pretreated with control small interfering RNA (siRNA) or PAR1-silencing RNA, and this was followed by a 30-min incubation with control or thrombin. Binding of monoclonal anti-PAR1 ATAP2 and SPAN12/5 was quantified in the absence and presence of the respective immunization peptide (blocking peptide). (B) ATAP2 and SPAN12/5 monoclonal anti-PAR1 binding was analyzed after a 3-h incubation with the indicated agonists. Background-subtracted normalized data are shown. (C) Cells were incubated for 30 min with the indicated concentrations of wild-type or γ-carboxyglutamic acid (Gla)-domain-deleted (desGla) proteases, and this was followed by analysis of SPAN12/5 binding. (D) Cells were incubated (30 min) with the indicated agonists in the absence and presence of the FXa inhibitor nematode anticoagulant protein 5 (NAP5) (1 μM), and this was followed by analysis of SPAN12/5 binding. Data are given as means ± standard errors of the mean. (A) $n = 4$; (B) $n = 5$; (C, D) $n = 6$. $^{**}P < 0.005$ as compared with control. APC, activated protein C.

tested whether FXa requires EPCR coreceptor binding in order to efficiently cleave PAR1. We first investigated whether monoclonal anti-EPCR prevents PAR1 cleavage. Both FXa and APC were found to efficiently cleave PAR1 in the absence of anti-EPCR and in the presence of the non-blocking anti-EPCR RCR92. In contrast, the blocking anti-EPCR RCR252 completely prevented the downregulation of SPAN12/5 binding by FXa and APC (Fig. 4A). Cleavage of PAR1 low-dose thrombin was not affected by anti-EPCR. Two different complementary approaches were used to independently confirm the surprising finding that EPCR is required for PAR1 cleavage by FXa in endothelial cells. First, we used siRNA to downregulate EPCR expression in EA.hy926 cells. Transcript levels for EPCR in the transfected cells were below 20% of those in control transfected cells under our experimental conditions, and surface protein expression was reduced by 60% (Fig. 4B). This moderate downregulation was associated with a highly significant decrease in the efficiency of PAR1 cleavage by FXa and APC, whereas the effects of thrombin and plasmin on SPAN12/5 binding were unchanged (Fig. 4C). Importantly, downregulation of EPCR did not affect native PAR1 expression. Second, we used a reporter construct that encodes AP fused to the N-terminus of human PAR1 to directly analyze PAR1 cleavage in HEK293 cells. Coexpression of EPCR led to significantly more efficient release of AP by FXa and APC from the reporter construct (Fig. 5A). This finding was not caused by increased surface expression of AP-tagged PAR1 in the EPCR-cotransfected cells, as shown in Fig. 5B. AP release by both proteases was dose-dependent, with significant effects being seen already at 12.5 nM (Fig. 5C). FXa and, to a lesser extent, APC also led to AP release in cells only transfected with PAR1, but both proteases were significantly more efficient if EPCR was also expressed. Thus, EPCR supports FXa-mediated cleavage of both endogenous endothelial PAR1 and overexpressed tagged PAR1 in the HEK293 cell system.

EPCR coreceptor binding does not affect FX activation

The endothelial cell surface is highly organized and compartmentalized, and we tested whether the interaction with EPCR might play a role in modulating activation of FX by the tissue factor–FVIIa complex. Quiescent EA.hy926 cells express only

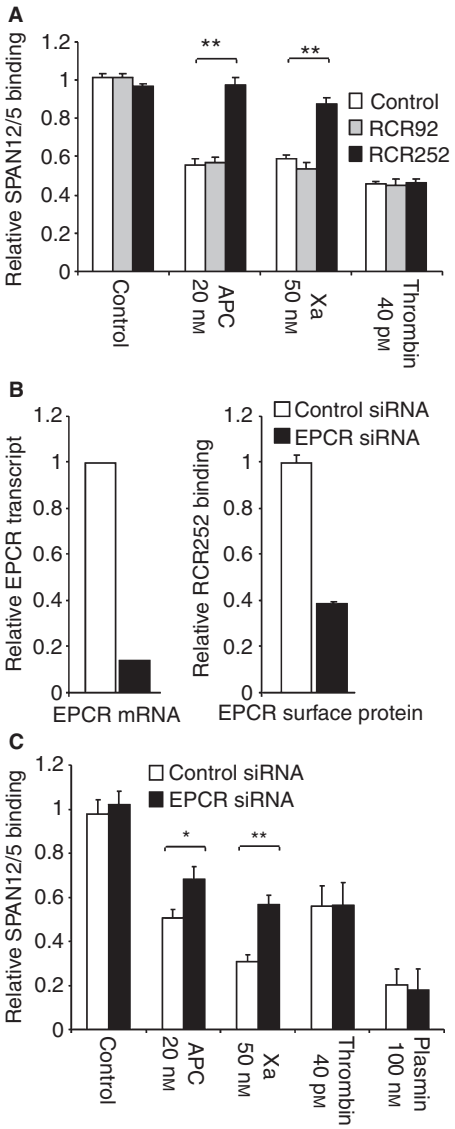

Fig. 4. Role of endothelial protein C receptor (EPCR) in the FXa mediated downregulation of SPAN12/5 binding to endothelial cells. (A) EA.hy926 cells were preincubated for 15 min in the absence or presence of 25 μg mL^{-1} monoclonal anti-EPCR RCR92 (non-blocking) or RCR252 (blocking), and this was followed by a 30-min incubation with the indicated agonists and quantification of cleavage-sensitive anti-PAR1 SPAN12/5 binding. (B) EA.hy926 cells were transfected with control small interfering RNA (siRNA) or siRNA targeting EPCR. EPCR transcript levels were analyzed by real-time polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase (left panel). Cell surface-expressed EPCR protein was quantified by immunoassay using monoclonal anti-EPCR RCR252 (right panel). (C) SPAN12/5 binding was quantified after a 30-min incubation with the indicated agonists in EA.hy926 cells transfected with either control or siRNA targeting EPCR. Data are given as means ± standard errors of the mean. [A, B (right panel), C] $n = 9$; [B (left panel)] $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.005$. APC, activated protein C.

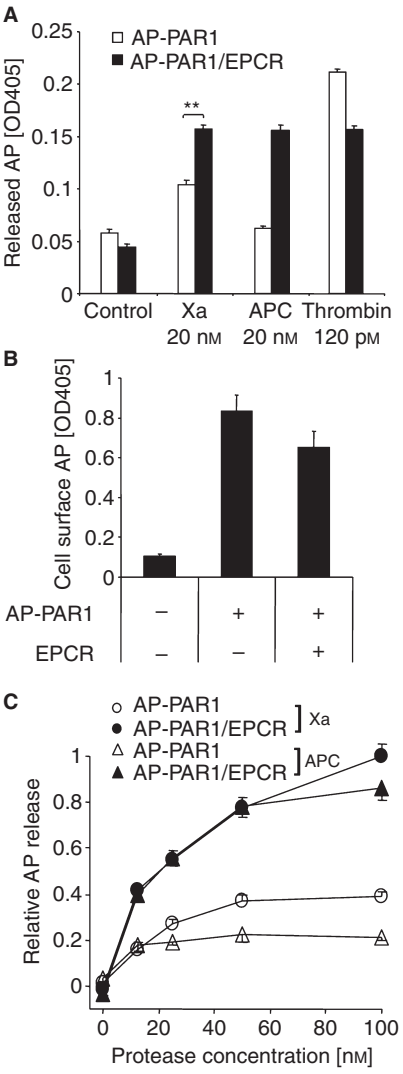

Fig. 5. Cleavage of overexpressed alkaline phosphatase (AP)-tagged protease-activated receptor-1 (PAR1) by FXa. (A) HEK293 cells were transiently transfected with human PAR1 tagged with AP at the N-terminus, either alone or together with human endothelial protein C receptor (EPCR). Cells were incubated with the indicated agonists for 20 min, and released AP activity was quantified in the conditioning medium. (B) Cell surface AP activity was quantified in HEK293 cells transfected as indicated. (C) HEK293 cells were transfected with AP–PAR1 and control or EPCR, and this was followed by incubation (20 min) with various concentrations of FXa or activated protein C (APC) as indicated. Released AP activity was analyzed. Data are given as mean ± standard error of the mean. (A) $n = 8$; (B) $n = 12$; (C) $n = 5$. $^{**}P < 0.005$.

minimal amounts of tissue factor, and were found to marginally support FXa generation, whereas tumor necrosis factor-α (TNF-α)-induced cells supported substantial FXa generation. Blocking of EPCR had no effect on FXa generation in quiescent or TNF-α-stimulated cells (Fig. 6A). EPCR binding of protein C increases activation efficiency by the thrombin–thrombomodulin complex, and it was recently reported that high concentrations of FVIIa can decrease protein C activation slightly by competing for EPCR binding [14]. We found that high concentrations of FXa, FVIIa (Fig. 6B) or APC–DEGR (not shown) do, indeed, all similarly interfere with APC generation, providing additional evidence that APC, FVIIa and FXa all compete for EPCR binding on the endothelial surface.

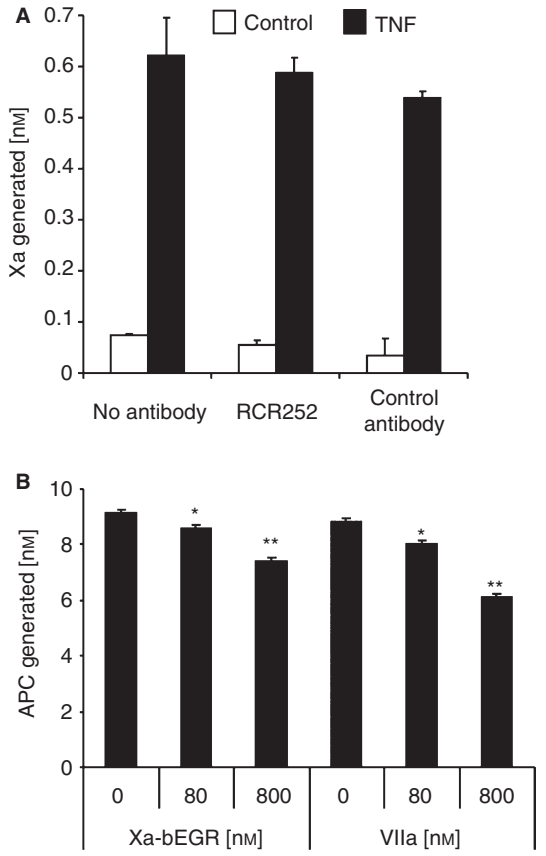

Fig. 6. Effect of FXa–endothelial protein C receptor (EPCR) interaction on FXa and activated protein C (APC) generation. (A) EA.hy926 cells were serum-starved for 5 h in the absence or presence of 1 nM tumor necrosis factor-α (TNF-α) and incubated with FVIIa (20 nM) and FX (100 nM) in the absence or presence of RCR252 or an isotype-matched non-specific control antibody (25 μg mL^{-1}). The generated FXa was quantified by amidolytic assay. (B) Cells were incubated with protein C (80 nM) and thrombin (1 nM) for 1 h in the presence of the indicated concentrations of competitors for EPCR binding, and this was followed by quantification of the generated APC. Data are given as mean ± standard error of the mean. $n = 3$, $*P < 0.05$ and $**P < 0.005$ as compared with no competitor. bEGR.

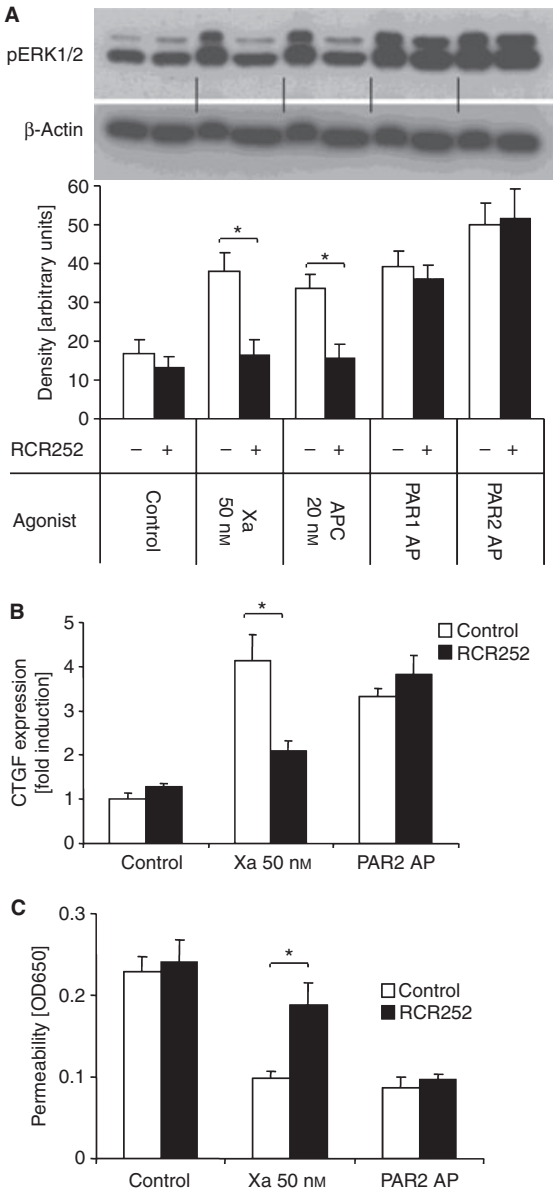

Fig. 7. Effect of blocking endothelial protein C receptor (EPCR) binding on downstream signaling by FXa in endothelial cells. (A) EA.hy926 cells were serum-starved in the absence or presence of a non-specific isotype-matched rat antibody or anti-EPCR RCR252 (both at 25 μg mL^{-1}), and this was followed by a 7-min incubation with the indicated proteases and agonist peptides [alkaline phosphatase (AP); protease-activated receptor-1 (PAR1) AP was TFLLRNPNDK at 20 μM; protease-activated receptor-2 (PAR2) AP was SLIGRL at 25 μM]. The upper part of the panel shows a representative immunoblot, and the lower part shows quantitative analyses of another set of four experiments. β-Actin was used as a loading control to normalize the quantitative results. (B) Connective tissue growth factor (CTGF) transcript expression was analyzed by real-time polymerase chain reaction after a 3-h incubation of EA.hy926 cells with the indicated agonists. Results were normalized to glyceraldehyde-3-phosphate dehydrogenase transcript levels. (C) Subconfluent cells in a Transwell chamber were incubated for 3 h with the indicated agonists, and permeability was determined as described in Materials and methods. Data are given as means ± standard errors of the mean. $n = 4$. $*P < 0.05$. APC, activated protein C; pERK, phosphorylated extracellular signal-related kinase.

EPCR binding is required for downstream signaling by FXa

We analyzed whether EPCR coreceptor binding is also required for downstream signaling responses to FXa in endothelial cells. Endothelial cells express both PAR1 and PAR2, and FXa has been shown to elicit signaling through both of these PARs. FXa and APC, as well as the PAR1-specific and PAR2-specific agonist peptides, substantially induced phosphorylation of ERK1/2 MAP kinase in EA.hy926 cells. If the cells were preincubated with EPCR-blocking antibodies, phospho-MAP kinase induction by FXa and APC was abolished, whereas responses to the agonist peptides were not affected, demonstrating that both PAR1 and PAR2 were still available for signal transduction (Fig. 7A). FXa signaling has been shown to induce a profibrotic response, including the induction of CTGF [27]. Induction of the CTGF transcript by FXa was strongly reduced in the presence of blocking anti-

EPCR (Fig. 7B). We have previously shown that FXa can mediate endothelial barrier protective signaling through either PAR1 or PAR2 signaling [26]. Blocking anti-EPCR significantly reduced barrier enhancement by FXa in a dual chamber assay (Fig. 7C). Thus, downstream signaling by FXa requires binding to EPCR in our assays.

Discussion

Using several approaches, that is, overexpression of EPCR in different cell lines, EPCR-blocking antibodies, and reducing endothelial cell surface expression of EPCR, we have demonstrated that EPCR supports surface binding, PAR1 cleavage, and signaling by FXa. EPCR was recently shown to also bind FVIIa [13–15], and our results add to accumulating evidence that this receptor is able to support cell recruitment and positioning of Gla-domain-containing proteins other than protein C. Previous studies analyzing binding of APC to the endothelial cell surface indicated that FX and FXa compete only very inefficiently for binding [28]. In contrast, recent results from Ghosh *et al*. [14] indicate that on the endothelial cell surface, FX and protein C are similarly efficient in competing for EPCR binding of FVIIa, and cocompetition of protein C plus FX did not additionally affect FVIIa binding, suggesting that APC, FVIIa and FX share binding sites. Interestingly, however, FX failed to compete for FVIIa binding to EPCR-transfected CHO-K1 cells in the study by Ghosh *et al*., whereas FX efficiently competed for FXa binding in our studies in stably transfected CHO-K1 cells (Fig. 1F). The Gla domains of both human protein C and FVII contain a leucine at position 8, and this residue has been shown to be crucial for EPCR recognition using purified proteins in surface plasmon resonance studies [13]. Given that human FX contains a methionine at this position, it is possible that EPCR is necessary but not sufficient for FXa binding, and that cell type-specific differences in the expression of other cofactor(s) that contribute to EPCR-dependent and EPCR-independent binding may explain the conflicting results in competition studies. One other such cofactor may be annexin 2, which was recently implicated as an endothelial cell receptor that supports PAR1-dependent signaling by FXa [12]. Another possibility is that different forms of EPCR itself have specific relative affinities for the various ligands. Extensive studies in purified systems, using EPCR from different sources, will be required to quantitatively establish binding affinities and competition efficiencies of the coagulation proteases for EPCR binding. Analysis of these data, together with results obtained using cell surface-expressed EPCR, may reconcile the discrepant results in the literature.

EPCR supports PAR1 activation by APC [6], and is known to colocalize with PAR1 in lipid rafts in the cell membrane [29]. Consistent with the concept that EPCR can recruit and position coagulation proteases in close proximity to PAR1 for efficient activation, our results directly demonstrate that cleavage of endothelial PAR1 by FXa depends to a large extent on the availability of EPCR binding. However, endothelial cells express high levels of both PAR1 and PAR2, and one fundamental difference between APC and FXa is that APC signaling seems to be completely PAR1-dependent, whereas FXa can activate both PAR1 and PAR2 on endothelial cells. Specifically, we have previously shown that FXa can mediate the induction of MAP kinase phosphorylation and barrier protection in EA.hy926 cells through either PAR1 or PAR2 [26]. Our present finding that both of these downstream responses to FXa in the same cell system depend on EPCR thus indicates that PAR2 activation by FXa also requires EPCR binding and suggests that EPCR also colocalizes with PAR2 in the cell surface. If this turns out to be the case, another question will be why endothelial PAR2 supports FXa signaling but not APC signaling, given that APC can activate PAR2 in an overexpression system [6]. One possibility is that post-translational modifications, for example glycosylation [30] of PAR2, play a role in restricting activation to certain proteases in endothelial cells. Clearly, further studies and novel tools will be required to directly analyze cleavage of endogenous PAR2 in endothelial cells in order to address these questions.

The physiological or pathophysiological role of FXa signaling remains to be established, even though a variety of cellular responses of potential relevance have been described in cell culture [10]. Our current study shows that EPCR binding plays an important role in PAR activation by FXa and both FXa's profibrotic effects (CTGF induction) and the potentially cytoprotective and anti-inflammatory barrier enhancement. It is possible that other cellular responses to FXa are EPCR-independent. However, PAR activation has been implicated in most FXa responses in cells, and it is therefore likely that expanding the scope of the present studies will lead to the discovery of other downstream effects of FXa that are EPCR-dependent. Relatively high FXa concentrations of unknown physiological relevance were used in most *in vitro* studies. However, these concentrations may be reached locally, resulting in FXa-dependent signaling, for example in the microenvironment of endothelial and tumor cells [31]. Many cancer cell lines are known to express not only PARs but also EPCR [32]. EPCR binding may not only play a key role in FXa signaling, but may also create a pool of FX/Xa on the cellular surface and affect the clearance and trafficking of this coagulation factor across cell barriers [33]. In conclusion, our results demonstrate that EPCR plays a key role in supporting cell surface recruitment, PAR1 cleavage and signaling by FXa in endothelial cells. Thus, EPCR constitutes an unexpected link where the protein C and FX pathways intersect. Further studies are needed to address the implications of this crosstalk for potential approaches to the therapeutic targeting of proinflammatory and anti-inflammatory cellular signaling by FXa and APC.

Acknowledgements

We thank K. Fukudome for generously providing us with large amounts of anti-EPCR antibodies, L. Brass for the SPAN12 hybridoma cells, L. Mosnier for the modified AP coding

sequence, and C. Lau for excellent technical assistance. This study was supported by National Institutes of Health grant HL 73318 (to M. Riewald) and grants from the Swiss Foundation for Medical-Biological Grants (SSMBS), as well as a fellowship from the Mach-Gaensslen Stiftung (to R. A. Schuepbach).

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

References

1 Edgington TS, Mackman N, Brand K, Ruf W. The structural biology of expression and function of tissue factor. *Thromb Haemost* 1991; **66**: 67–79.
2 Broze GJ Jr. Tissue factor pathway inhibitor and the revised theory of coagulation. *Annu Rev Med* 1995; **46**: 103–12.
3 Esmon CT. The protein C pathway. *Chest* 2003; **124**: 26S–32S.
4 Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost* 2005; **3**: 1800–14.
5 Camerer E, Huang W, Coughlin SR. Tissue factor- and factor X-dependent activation of protease-activated receptor 2 by factor VIIa. *Proc Natl Acad Sci USA* 2000; **97**: 5255–60.
6 Riewald M, Petrovan RJ, Donner A, Mueller BM, Ruf W. Activation of endothelial cell protease activated receptor 1 by the protein C pathway. *Science* 2002; **296**: 1880–2.
7 Mosnier LO, Zlokovic BV, Griffin JH. The cytoprotective protein C pathway. *Blood* 2007; **109**: 3161–72.
8 Esmon CT, Xu J, Gu JM, Qu D, Laszik Z, Ferrell G, Stearns-Kurosawa DJ, Kurosawa S, Taylor FB Jr, Esmon NL. Endothelial protein C receptor. *Thromb Haemost* 1999; **82**: 251–8.
9 Oganesyan V, Oganesyan N, Terzyan S, Qu D, Dauter Z, Esmon NL, Esmon CT. The crystal structure of the endothelial protein C receptor and a bound phospholipid. *J Biol Chem* 2002; **277**: 24851–4.
10 Borensztajn K, Peppelenbosch MP, Spek CA. Factor Xa: at the crossroads between coagulation and signaling in physiology and disease. *Trends Mol Med* 2008; **14**: 429–40.
11 Riewald M, Ruf W. Mechanistic coupling of protease signaling and initiation of coagulation by tissue factor. *Proc Natl Acad Sci USA* 2001; **98**: 7742–7.
12 Bhattacharjee G, Ahamed J, Pawlinski R, Liu C, Mackman N, Ruf W, Edgington TS. Factor Xa binding to annexin 2 mediates signal transduction via protease-activated receptor 1. *Circ Res* 2008; **102**: 457–64.
13 Preston RJ, Ajzner E, Razzari C, Karageorgi S, Dua S, Dahlback B, Lane DA. Multifunctional specificity of the protein C/activated protein C Gla domain. *J Biol Chem* 2006; **281**: 28850–7.
14 Ghosh S, Pendurthi UR, Steinoe A, Esmon CT, Rao LV. Endothelial cell protein C receptor acts as a cellular receptor for factor VIIa on endothelium. *J Biol Chem* 2007; **282**: 11849–57.
15 Lopez-Sagaseta J, Montes R, Puy C, Diez N, Fukudome K, Hermida J. Binding of factor VIIa to the endothelial cell protein C receptor reduces its coagulant activity. *J Thromb Haemost* 2007; **5**: 1817–24.
16 Schuepbach RA, Feistritzer C, Brass LF, Riewald M. Activated protein C-cleaved protease activated receptor-1 is retained on the endothelial cell surface even in the presence of thrombin. *Blood* 2007; **111**: 2667–73.
17 Brass LF, Pizarro S, Ahuja M, Belmonte E, Blanchard N, Stadel JM, Hoxie JA. Changes in the structure and function of the human thrombin receptor during receptor activation, internalization, and recycling. *J Biol Chem* 1994; **269**: 2943–52.
18 Ye X, Fukudome K, Tsuneyoshi N, Satoh T, Tokunaga O, Sugawara K, Mizokami H, Kimoto M. The endothelial cell protein C receptor (EPCR) functions as a primary receptor for protein C activation on endothelial cells in arteries, veins, and capillaries. *Biochem Biophys Res Commun* 1999; **259**: 671–7.
19 Feistritzer C, Schuepbach RA, Mosnier LO, Bush LA, Di Cera E, Griffin JH, Riewald M. Protective signaling by activated protein C is mechanistically linked to protein C activation on endothelial cells. *J Biol Chem* 2006; **281**: 20077–84.
20 Stassens P, Bergum PW, Gansemans Y, Jespers L, Laroche Y, Huang S, Maki S, Messens J, Lauwereys M, Cappello M, Hotez PJ, Lasters I, Vlasuk GP. Anticoagulant repertoire of the hookworm Ancylostoma caninum. *Proc Natl Acad Sci USA* 1996; **93**: 2149–54.
21 Edgell CJ, McDonald CC, Graham JB. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc Natl Acad Sci USA* 1983; **80**: 3734–7.
22 Feistritzer C, Riewald M. Endothelial barrier protection by activated protein C through PAR1-dependent sphingosine 1-phosphate receptor-1 crossactivation. *Blood* 2005; **105**: 3178–84.
23 Mosnier LO, Zampolli A, Kerschen EJ, Schuepbach RA, Banerjee Y, Fernandez JA, Yang XV, Riewald M, Weiler H, Ruggeri ZM, Griffin JH. Hyper-antithrombotic, non-cytoprotective Glu149Ala-activated protein C mutant. *Blood* 2009; **113**: 5970–8.
24 Schuepbach RA, Feistritzer C, Fernandez JA, Griffin JH, Riewald M. Protection of vascular barrier integrity by activated protein C in murine models depends on protease-activated receptor-1. *Thromb Haemost* 2009; **101**: 724–33.
25 Riewald M, Ruf W. Protease-activated receptor-1 signaling by activated protein C in cytokine-perturbed endothelial cells is distinct from thrombin signaling. *J Biol Chem* 2005; **280**: 19808–14.
26 Feistritzer C, Lenta R, Riewald M. Protease-activated receptors-1 and -2 can mediate endothelial barrier protection: role in factor Xa signaling. *J Thromb Haemost* 2005; **3**: 2798–805.
27 Riewald M, Kravchenko VV, Petrovan RJ, O'Brien PJ, Brass LF, Ulevitch RJ, Ruf W. Gene induction by coagulation factor Xa is mediated by activation of protease-activated receptor 1. *Blood* 2001; **97**: 3109–16.
28 Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem* 1994; **269**: 26486–91.
29 Bae JS, Yang L, Rezaie AR. Receptors of the protein C activation and activated protein C signaling pathways are colocalized in lipid rafts of endothelial cells. *Proc Natl Acad Sci USA* 2007; **104**: 2867–72.
30 Compton SJ, Renaux B, Wijesuriya SJ, Hollenberg MD. Glycosylation and the activation of proteinase-activated receptor 2 (PAR(2)) by human mast cell tryptase. *Br J Pharmacol* 2001; **134**: 705–18.
31 Morris DR, Ding Y, Ricks TK, Gullapalli A, Wolfe BL, Trejo J. Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration, and invasion of breast cancer cells. *Cancer Res* 2006; **66**: 307–14.
32 Tsuneyoshi N, Fukudome K, Horiguchi S, Ye X, Matsuzaki M, Toi M, Suzuki K, Kimoto M. Expression and anticoagulant function of the endothelial cell protein C receptor (EPCR) in cancer cell lines. *Thromb Haemost* 2001; **85**: 356–61.
33 Nayak RC, Sen P, Ghosh S, Gopalakrishnan R, Esmon CT, Pendurthi UR, Rao LV. Endothelial cell protein C receptor cellular localization and trafficking: potential functional implications. *Blood* 2009; **114**: 1974–86.